National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service

Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

NL-339 (r. 88/04) c

Canada

UNIVERSITY OF ALBERTA

ANALYSIS AND METABOLISM OF TRIMIPRAMINE, IPRINDOLE AND IMIPRAMINE IN MAMMALS

BY ©

MOHAMMED SALEH HUSSAIN

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND
RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF
DOCTOR OF PHILOSOPHY

IN

PHARMACEUTICAL SCIENCES (DRUG METABOLISM)

FACULTY OF PHARMACY AND PHARMACEUTICAL SCIENCES

EDMONTON, ALBERTA, CANADA

SPRING 1992

National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service

Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-73126-5

Canada

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: Mohammed Saleh Hussain

TITLE OF THESIS: Analysis and metabolism of trimipramine, iprindole and imipramine in mammals

DEGREE: Doctor of Philosophy

YEAR THIS DEGREE GRANTED: Spring, 1992

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as hereinbefore provided neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.

(Signed) M. S. Hussain

437 Michener Park
Edmonton, Alberta
Canada T6H 4M5

February 6, 1992

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled ANALYSIS AND METABOLISM OF TRIMIPRAMINE, IPRINDOLE AND IMIPRAMINE IN MAMMALS submitted by MOHAMMED SALEH HUSSAIN in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in PHARMACEUTICAL SCIENCES (DRUG METABOLISM).

RT Coutts

Dr. Ronald T. Coutts
(Supervisor)

M. Daneshtalab

Dr. Mohsen Daneshtalab

Dr. Ronald G. Micetich

Dr. Glen B. Baker

Dr. Leonard I. Wiebe

Dr. Gordon McKay
(External Examiner)

February 6, 1992

This thesis is dedicated to my wife,

Khushi,

who has given me love, support and encouragement
throughout my studies.

In the name of God, Most Gracious, Most Merciful.

Read in the name of thy Lord and Cherisher, Who created man out of a clot of congealed blood. Read and thy Lord is Most Bountiful, He who taught the use of the Pen and Taught man that which he knew not. (Qur'an, 96:1-5)

ABSTRACT

The tricyclic antidepressant (TCA) drug trimipramine has been used clinically for several years. Few data about its metabolism in human are available. Much less is known about the mechanism of action of trimipramine than of the other TCAs. Therefore, a knowledge of its metabolism in man, and in suitable animal models, is essential to the understanding of the pharmacology of trimipramine.

Little is known regarding the involvement of the cytochrome P450 isozyme in the metabolic oxidation of trimipramine. One method of delineating the P450 isozyme(s) that is (are) involved in drug metabolism reactions is to administer a known inhibitor of metabolism during, or prior to, administration of the drug of interest. Prior to the commencement of such studies involving trimipramine in the rat, a knowledge of its metabolism in that species was required. Therefore, a study was designed and 20 urinary metabolites of trimipramine were identified in the rat, of which 12 were novel. The metabolism of trimipramine in human was also studied and 10 metabolites were characterized, including 3 novel ones.

To investigate the P450 isozyme responsible for metabolic oxidation of trimipramine, drug-drug interaction studies involving TMP, quinidine, quinine and iprindole were designed. The results of the studies indicated that P450IID6

isozyme was involved in the aromatic hydroxylation of trimipramine.

There is a paucity of information about the metabolism of iprindole in different species. Therefore, studies were designed to characterize the urinary and biliary metabolites of iprindole in the rat and it was observed that iprindole underwent a significant biliary excretion in that species.

Metabolism of imipramine in human was conducted to investigate the contribution of N,N-didemethylation in the overall metabolism of imipramine. The results showed that this was a minor metabolic pathway in that subject.

A study was also designed to determine the contribution of the P450IID6 isozyme in the aromatic hydroxylation of N-*n*-butylamphetamine. The conclusion from this study was that aromatic hydroxylation of N-*n*-butylamphetamine is catalyzed by a P450 isozyme other than P450IID6.

A simple and rapid GC analytical method was also developed to determine the hepatic P450IID6 activity in humans.

ACKNOWLEDGEMENTS

The author wishes to express sincere thanks to Dr. Ronald T. Coutts, Dr. Ronald G. Micetich and Dr. Mohsen Daneshtalab for their support and keen interest to the research undertaken.

Dr. R.T. Coutts's availability, guidance, stimulating encouragement throughout the course of this study are greatly appreciated. Dr. Coutts's friendly and excellent supervision enabled the author to carry on with this study in an environment coducive to research and learning.

The author is indebted to Dr. R.G. Micetich for his inspiration, confidence and continued financial support throughout the duration of the study. Without his financial help this research would not have been possible. Dr. Daneshtalab's constant encouragement and friendly advice during the past few years are also greatly appreciated.

The author would also like to thank Dr. Glen B. Baker for his patience in correcting this thesis and for providing several authentic metabolites for this research work. His friendly attitude, constant inspiration and suggestions were invaluable to the author.

Sincere thanks are also extended to Dr. Leonard I. Wiebe for his support, advice and assistance over the past three years.

The author wishes to thank Dr. F.M. Pasutto for his continued encouragement and stimulating criticism during the course of this research work.

Appreciation is also extended to Dr. Mathew Martin-Iversion and Mr. Lakhdar Aggoun for their help in the statistical analyses.

A large portion of the credit for the timely completion of this research goes to Mr. Don Whyte and Mr. Zaher Samnani for their technical assistance. The author is indebted to Mr. Don Whyte for his valuable help during this thesis research. His hard work, technical expertise and commitment to excellence paid valuable dividends in both the field and in the laboratory.

The author gratefully acknowledged the financial assistance from the following organizations:

1. SynPhar Laboratories Inc., Edmonton
2. Alberta Heritage Foundation for Medical Research (AHFMR full-time studentship award)
3. Myer Horowitz graduate scholarship
4. Faculty of Graduate Studies and Research, University of Alberta (Travel grants)
5. Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta (Graduate Teaching Assistantship)
6. University of Alberta Bursary Award

The author very much appreciate the cooperation and friendship of the fellow graduate students and the staff of the faculty of pharmacy and pharmaceutical Sciences, University of Alberta which made author's sojourn here an enjoyable one.

Finally, and most importantly, are the thanks to Khushi, Shakir, Sufi, Sarah and my dearest parents for their unending patience, love and support.

TABLE OF CONTENTS

CHAPTER PAGE

1. INTRODUCTION 1
  1.1 The Drugs 3
    1.1.1 Trimipramine (TMP) 3
      1.1.1.1 Physicochemical properties 3
      1.1.1.2 Pharmacology 5
      1.1.1.3 Side effects and toxicity 6
    1.1.2 Iprindole (IPR) 7
      1.1.2.1 Physicochemical properties 7
      1.1.2.2 Pharmacology 8
      1.1.2.3 Side effects and toxicity 10
  1.2 Metabolism 10
    1.2.1 Phase I metabolic reactions 13
      1.2.1.1 The Cytochrome P450 isozyme systems 18
    1.2.2 Phase II metabolic reactions 24
    1.2.3 Metabolism of tricyclic antidepressants (TCAs) 27
      1.2.3.1 Active metabolites of TCAs 35
  1.3 Analysis of TCAs 38
  1.4 Enzyme induction and inhibition 39
  1.5 Polymorphism in drug oxidation and phenotyping 43
    1.5.1 Sparteine and debrisoquine 45
    1.5.2 Mephenytoin 49
    1.5.3 Phenotyping 52
      1.5.3.1 Debrisoquine (DQN) 52
      1.5.3.2 Sparteine (SPT) 53
      1.5.3.3 Dextromethorphan (DM) 54
  1.6 Analytical Procedures 56

1.6.1 Isolation and purification of drugs and their metabolites from biological fluids 56

1.6.2 Derivatization technique 57

1.6.3 Gas chromatography 59

1.6.4 Gas chromatography-Mass spectrometry 61

1.7 *In vitro* and *in vivo* animal models of drug metabolism 62

1.7.1 *In vitro* models 62

1.7.2 *In vivo* animal models 67

1.7.2.1 Intact animals 67

1.7.2.2 Bile duct cannulated animals 68

2. RATIONALE, HYPOTHESES, AIMS AND OBJECTIVES 70

2.1 Rationale for the thesis research 70

2.1.1 Trimipramine 70

2.1.2 Drug-drug interaction studies 71

2.1.3 Iprindole 71

2.1.4 Biliary excretion studies 72

2.2 Hypotheses 73

2.3 Aims and objectives 74

3. MATERIALS AND METHODS 77

3.1 Chemicals and biologicals 77

3.1.1 Commercial sources 77

3.1.2 Gifts 78

3.1.3 Distilled water 79

3.1.4 Solvent distillation 79

3.2 Instrumentation 79

3.2.1 Gas chromatography 79

3.2.1.1 Nitrogen phosphorus detection 79

3.2.1.2 Flame ionization detection 79

3.2.1.3 Electron capture detection 80

3.2.1.4 Mass spectrometer detection 80

3.2.1.5 GC columns 80

3.2.2 Gas chromatography-Mass spectrometry 81

3.2.3 Tissue homogenizer 82
3.2.4 Centrifuges 82
3.2.5 Shaker-Mixer 83
3.2.6 Other instruments 83
3.4 Glassware cleaning 83
3.5 Animals 84
3.6 Liver samples 85
3.7 *In vitro* studies 85
3.7.1 Preparation of rat liver microsomes 85
3.7.2 Incubation procedure 85
3.8 Bile duct cannulation 86
3.9 Derivatization techniques general method 87
3.9.1 Acidic metabolites 87
3.9.2 Basic metabolites 88
3.10 TMP experimental 88
3.10.1 Extraction of TMP maleate from Surmontil® tablets 88
3.10.2 Dose and sample collection 89
3.10.3 Isolation of TMP and its metabolites from liver tissues 91
3.10.4 Isolation of TMP and metabolites from urine samples 92
3.10.4.1 Hydrolysis procedure 92
3.10.4.2 Extraction of acidic metabolites 92
3.10.4.3 Extraction of basic metabolites 93
3.10.5 Enzyme hydrolysis and extraction of metabolites from bile samples 94
3.10.5.1 Isolation of total (phase I and phase II) metabolites 94
3.10.5.2 Isolation of phase I metabolites 94
3.10.5.3 Hydrolysis and extraction of phase II metabolites 95
3.10.6 Assay procedure 96
3.11 Nortrimipramine (norTMP) and 2-hydroxy trimipramine (2-OH-TMP) experimental 97

3.11.1 Dose and sample collection 97
3.11.2 Hydrolysis and extraction procedure 98
3.11.3 Gas chromatography 98
3.12 IPR experimental 98
3.12.1 Treatment of animals 98
3.12.2 Incubation and extraction procedure 99
3.12.3 Isolation of IPR metabolites from liver tissues 100
3.12.4 Extraction of IPR metabolites from rat bile sample 100
3.12.5 Analytical method 101
3.13 Imipramine (IMI) experimental 101
3.13.1 Dose and sample collection 101
3.13.1.1 Human study 101
3.13.1.2 Biliary excretion study 102
3.13.2 Hydrolysis and extraction procedures 102
3.13.2.1 Human urine sample 102
3.13.2.2 Rat bile sample 103
3.13.3 Assay method 103
3.14 N-n-butylamphetamine (NBA) experimental 104
3.14.1 Animals and treatment 104
3.14.2 Extraction procedure 105
3.14.3 Analytical procedure 105
3.15 Phenotyping experimental 106
3.15.1 Subjects and method 106
3.15.2 Hydrolysis and extraction procedure 106
3.15.3 Gas chromatography 107
3.16 Calibration graphs 108
3.16 Statistical analyses 108

4. RESULTS AND DISCUSSION 110
4.1 Studies conducted on TMP 110
4.1.1 *In vivo* metabolism studies 110
4.1.1.1 Metabolic studies of TMP in the rat 110

        - 4.1.1.2 Biliary excretion study 129
        - 4.1.1.3 Metabolism of TMP in man 131
    - 4.1.2 *In vitro* metabolism studies of TMP 134
        - 4.1.2.1 Rat liver microsomes 134
    - 4.1.3 Drug-drug interaction studies 163
        - 4.1.3.1 Effects of IPR on the metabolism of TMP 163
        - 4.1.3.2 Effects of QND on the metabolism of TMP 171
        - 4.1.3.3 Effects of QNN on the metabolism of TMP 175
- 4.2 Studies conducted on 2-OH-TMP 178
    - 4.2.1 Urinary metabolites of 2-OH-TMP in the rat 178
- 4.3 Studies conducted on norTMP 180
    - 4.3.1 *In vivo* metabolic study in rat 180
    - 4.3.2 Effects of QND on the metabolism of norTMP 181
- 4.4 Studies conducted on IPR 183
    - 4.4.1 *In vivo* metabolism study 183
        - 4.4.1.1 Mass spectral observations of general application 184
        - 4.4.1.2 Identification of site of metabolic aliphatic (or alicyclic) oxidation 190
        - 4.4.1.3 GC-MS spectral studies 193
        - 4.4.1.4 Structure elucidation 201
    - 4.4.2 Biliary excretion study 209
    - 4.4.3 *In vitro* metabolic studies 210
        - 4.4.3.1 Rat liver microsomes 210
- 4.5 Studies conducted on NBA 213
    - 4.5.1 Effects of QND on the metabolism of NBA in the rat 213
- 4.6 Studies conducted on IMI 222
    - 4.6.1 Metabolism of IMI in human 222

4.6.1.1 Quantitation of IMI and metabolites in human urine 232
4.6.2 Biliary excretion study 233
4.7 Determination of hepatic cytochrome P450IID6 activity in human 245

5. SUMMARY AND CONCLUSIONS 252
5.1 Studies conducted on TMP 252
5.2 Studies conducted on IPR 256
5.3 Studies conducted on NBA 258
5.4 Studies conducted on IMI 258
5.5 Phenotyping experiment 259

6. FUTURE PERSPECTIVES 261

BIBLIOGRAPHY 264

APPENDIX:CURRICULUM VITAE 294

LIST OF FIGURES

Figure 1-1. Chemical structure of trimipramine (TMP) 4
Figure 1-2. Chemical structure of iprindole (IPR) 9
Figure 1-3. Metabolic formation of hippuric acid 12
Figure 1-4. Cytochrome P450-mediated carbon-oxidation cycle. 14
Figure 1-5. Schematic representation of some phase II metabolic reactions. 25
Figure 1-6. Structure of amitriptyline (AMI) 29
Figure 1-7. Structure of nortriptyline (NT) 30
Figure 1-8. Structure of imipramine (IMI) 31
Figure 1-9. Structure of clomipramine (CMI) 32
Figure 1-10. Structure of protriptyline (PROT) 33
Figure 1-11. Metabolism of S-mephenytoin in man. 50
Figure 1-12. Chemical structures of mephobarbital, tranylcypromine and phenytoin. 51
Figure 1-13. Chemical structures of debrisoquine (DQN) and its major metabolite, 4-hydroxy-debrisoquine 53
Figure 1-14. Metabolic pathway of sparteine in man 54
Figure 1-15. Structures of dextromethorphan (DM) and its major metabolite dextrorphan (DR) 55
Figure 1-16. Principal types of derivatization procedures used in GC analyses. 60
Figure 1-17. Schematic representation of the procedure used to prepare various fractions of liver homogenate for use in *in vitro* drug metabolism studies. 66
Figure 4-1. Total ion current chromatogram of an underivatized urine extract from a rat dosed intraperitoneally with trimipramine. 137

Figure 4-2. Total ion current chromatogram of an acetylated urine extract from a rat dosed intraperitoneally with trimipramine. 137
Figure 4-3. Mass spectra and structures of TMP and its underivatized and acetylated metabolites. 138
Figure 4-4. Mass fragmentation pathways of TMP and norTMP. 146
Figure 4-5. Structures of major diagnostic fragment ions of TMP and its metabolites. 147
Figure 4-6. Structures of urinary metabolites of TMP in the rat. 151
Figure 4-7. Structures of the biliary metabolites of TMP in the rat. 152
Figure 4-8. Typical GC traces of bile extracts from rats dosed intraperitoneally with trimipramine. 153
Figure 4-9. Total ion chromatogram of an underivatized urine extract from man dosed orally with trimipramine. 154
Figure 4-10. Structures of urinary metabolites of TMP in man. 155
Figure 4-11. Electron ionization mass spectrum of N-acetyl-2-OH-N,N-didesmethyl-TMP. 156
Figure 4-12. Mass fragmentation pathway of N-acetyl-2-OH-N,N-didesmethyl-TMP. 157
Figure 4-13. Structures of *in vitro* metabolites of TMP in the rat. 158
Figure 4-14a. Kinetics of formation of 2-OH-norTMP relative to norTMP from TMP in rat liver microsomes. Metabolites are assayed gas chromatographically at different time intervals. 159
Figure 4-14b. Kinetics of formation of 2-OH-norTMP relative to 2-OH-TMP from TMP in rat liver microsomes. 160

Figure 4-14c. Kinetics of formation of 10-OH-norTMP relative to norTMP from TMP in rat liver microsomes. 161
Figure 4-14d. Kinetics of formation of 10-OH-norTMP relative to 10-OH-TMP from TMP in rat liver microsomes. 162
Figure 4-15. Typical GC traces 169
Figure 4-16. Recoveries of TMP and metabolites from rat urine (72h) [Mean ± S.E.M.]. 170
Figure 4-17. Recoveries of TMP and metabolites from rat urine (72h) [Mean ± S.E.M.]. 174
Figure 4-18. Urinary excretion (72h) of TMP and metabolites in rat [Mean ± S.E.M.]. 177
Figure 4-19. Recoveries of norTMP and its metabolite from rat urine (72h) [Mean ± S.E.M.]. 182
Figure 4-20. Mass fragmentation pathways of iprindole (IPR). 186
Figure 4-21. Fragmentation pathways of N-demethylated and N,N-didemethylated IPR acetate. 187
Figure 4-22a. Expulsion of a ketene molecule from a cyclic phenolic acetate. 189
Figure 4-22b. Expulsion of an acetic acid molecule from a cyclic alcoholic acetate. 189
Figure 4-23a. Loss of acetic acid molecule from 2-acetoxy-1-phenylpropane. 190
Figure 4-23b. GC-MS behavior of 1-acetoxy-1-phenylpropane. 191
Figure 4-23c. Loss of acetic acid molecule from cholesterol acetate. 191
Figure 4-23d. Loss of acetic acid molecule from β-sitosterol acetate. 192
Figure 4-23e. GC-MS behavior of ethynodiol diacetate. 192
Figure 4-24. Total ion current chromatogram of a non-acetylated urine extract from a rat dosed intraperitoneally with iprindole. 194

Figure 4-25. Total ion current chromatogram of an acetylated urine extract from a rat dosed intraperitoneally with iprindole. 195
Figure 4-26. Proposed structures of IPR metabolites in rat urine. 196
Figure 4-27. Structures of fragment ions of IPR metabolites. 197
Figure 4-28. Structures of the biliary metabolites of IPR in the rat. 211
Figure 4-29. Structures of *in vitro* metabolites of IPR in the rat. 212
Figure 4-30. Chemical structures of NBA, 4-OH-NBA and 4-OH-3-OCH_3-NBA. 219
Figure 4-31. GC trace of an urine extract from a rat treated with N-(*n*-butyl)amphetamine. 220
Figure 4-32. Urinary excretion (48h) of N-(*n*-butyl) amphetamine and metabolites in the rat [Mean ± S.E.M.]. 221
Figure 4-33. Total ion current chromatogram of an underivatized urine extract from a female patient dosed po with imipramine. 234
Figure 4-34. Total ion current chromatogram of an acetylated urine extract from a female patient dosed po with imipramine. 235
Figure 4-35. Mass spectral fragmentation patterns of IMI and DMI. 236
Figure 4-36. Mass spectra and structures of IMI and its underivatized and acetylated metabolites. 237
Figure 4-37. Structures of major diagnostic fragment ions of IMI and its metabolites. 242
Figure 4-38. Structures of biliary metabolites of IMI in rat. 244
Figure 4-39. A typical GC trace of an urine extract from a subject (K019) treated with dextromethorphan. 248

LIST OF TABLES

Table 1-1. Examples of phase I reactions 16
Table 1-2. Human cytochrome P450 isozymes 20
Table 1-3. Animal cytochrome P450 isozymes 21
Table 1-4. Examples of phase II reactions 26
Table 1-5. List of some clinically used drugs known to be metabolized by P450IID6 isozyme 47
Table 1-6. List of some clinically used drugs known not to be metabolized by P450IID6 isozyme 49
Table 1-7. List of some drugs known to be metabolized by P450IIC9 isozyme. 51
Table 4-1. Mass spectral data on iprindole metabolites in rat 198

LIST OF ABBREVIATIONS

Amitriptyline	AMI
Amphetamine	AMP
Antidepressant	AD
Atomic mass unit	amu
Central nervous system	CNS
Centimeter	cm
Clomipramine	CMI
Cytochrome P450	P450
Debrisoquine	DQN
Degree celsius	°C
Desipramine	DMI
Desmethylnortriptyline	DNT
Desmethylclomipramine	DCMI
Dextromethorphan	DM
Dextrorphan	DR
Didesmethylimipramine	DDMI
Doxepin	DOX
Electron capture detector	ECD
Electron ionization	EI
Electron ionization mass spectrum(a)	EIMS
Electron volt	eV
Extensive metabolizer	EM
Flame ionization detector	FID
Gas chromatography	GC
Gas chromatography with flame-ionization detection	GC-FID
Gas chromatography with nitrogen-phosphorus detection	GC-NPD
Gas chromatography-mass spectrometry	GC-MS
Glucose-6-Phosphate	G6P
Hour(s)	h
High performance liquid chromatography	HPLC
4-Hydroxydebrisoquine	4-OH-DQN

2-Hydroxydesipramine	2-OH-DMI
10-Hydroxydesipramine	10-DMI
2-Hydroxyimipramine	2-OH-IMI
10-Hydroxyimipramine	10-OH-IMI
10-Hydroxynortrimipramine	10-OH-norTMP
2-Hydroxynortrimipramine	2-OH-norTMP
2-Hydroxytrimipramine	2-OH-TMP
5-Hydroxytryptamine (Serotonin)	5HT
Iminodibenzyl	IDB
Imipramine	IMI
Internal diameter	i.d.
Intraperitoneal	ip
Iprindole	IPR
Kilogram	kg
Male Sprague-Dawley	MSD
Mass Spectrometry	MS
Mass to charge ratio	m/z
Metabolic ratio	MR
Methoxyphenamine	MPA
3-Methylcholanthrene	3-MC
Microgram	µg
Microlitre	µl
Micrometer	µm
Milligram	mg
Millilitre	ml
Minute(s)	min
Mixed function oxidase	MFO
Molar	M
Molecular ion	$M^{+\cdot}$
Monoamine oxidase	MAO
Monoamine oxidase Inhibitor	MAOI
Nanogram	ng
Nanomole	n mole
N-*n*-Butylamphetamine	NBA
N-Ethylamphetamine	NEA
N-Methylamphetamine	NMA

N-*n*-Propylamphetamine	NPA
Nicotinamide adenine dinucleotide phosphate	NADP
Nitrogen phosphorus detector	NPD
Norepinephrine	NE
Nortrimipramine	norTMP
Nortriptyline	NT
Oral	po
Outer diameter	o.d.
Phenelzine	PZN
Phenobarbital	PB
Poor metabolizer	PM
Protriptyline	PROT
Quinidine	QND
Quinine	QNN
Reduced NADP	NADPH
Sparteine	SPT
Tranylcypromine	TCP
Tricyclic antidepressant	TCA
Trimipramine	TMP

1. INTRODUCTION

Depression is an occasional component of daily life, but it may also be a symptom of primary affective illness. Descriptions of depression are found even in ancient literature; early physicians associated melancholy with brain function. Although depression has been known since antiquity, the modern era in the treatment of depression began in the late 1950s with the almost simultaneous introduction of the tricyclic antidepressant (TCA) drug imipramine(IMI) and the monoamine oxidase inhibitor (MAOI) iproniazid (Klerman, 1984; Cooper, 1988). Both discoveries were serendipitous and resulted from clinical observations rather than laboratory experiments. IMI was evaluated as an antipsychotic agent but subsequently it was found to be effective in the treatment of depression. Likewise, iproniazid was initially used in the treatment of tuberculosis, but latter on it was found to be a potent MAOI. The introduction of the biogenic amine theory (Alpers and Himwich, 1969; Axelrod, 1971) of depression further stimulated the interest in antidepressant (AD) drugs. Accordingly, several TCA compounds were introduced in the 1960s and all of them had some effect on the presynaptic uptake of either norepinephrine (NE) or serotonin (5-hydroxytryptamine;5HT).

ADs containing a system of 3 fused rings in their structure are called TCAs. TCAs currently available in Canada include IMI, desipramine (DMI), trimipramine (TMP), clomipramine (CMI), amitriptyline (AMI), nortriptyline (NT), protriptyline (PROT) and doxepin (DOX). Iprindole (IPR), a second generation AD, is widely used in Europe. Examples of second generation ADs which have been more recently introduced on the Canadian market include fluvoxamine, fluoxetine, amoxapine, trazodone and maprotiline (Krogh *et al.*, 1991).

Depression is one of the most common psychiatric disorders. Depression, as an illness, will affect one out of seven North Americans during a lifetime (Lapierre, 1991). Its major complication, suicide, is among the leading causes of death in most western societies. About 75,000 Americans die annually by suicide. In a recent survey (Stinson, 1990) it was found that depression afflicts 11 million Americans, out of 27 million who suffer from mental disorders.

The search for new ADs has not yet resulted in any significant improvement in response rates, but has produced drugs that are more tolerable to patients *e.g.* fluoxetine, fluvoxamine and the most recent one, sertraline. These drugs are all 5-HT uptake inhibitors. Research is now in progress in different parts of the world to develop newer ADs with better response rates and fewer side effects.

1.1 The Drugs

1.1.1 Trimipramine (TMP)

1.1.1.1 Physicochemical properties

The TCA drug TMP was approved for use in USA in 1979, but it has been used in Europe since 1960, and in Canada prior to 1979. It is as effective as other antidepressants (Abernethy *et al.*, 1984; Assalian *et al.*, 1985) and its anxiolytic and sedative effects enhance its clinical utility (Lapierre, 1989). A common dose is 75-300 mg/day. Onset of its antidepressant action is generally delayed for 2-4 weeks.

TMP maleate is a white crystalline powder, melting point 140-144°C, with a molecular weight (MW) of 410.5 (TMP base, MW=294.42). It is moderately soluble in water and ethanol but freely soluble in chloroform (Moffat *et al.*, 1986). The chemical structure of TMP, 5-(3-dimethylamino-2-methyl propyl)-10,11-dihydro-5H-dibenz[b,f]azepine, is depicted in Figure 1-1. An analog of IMI, possessing a chiral center at 2′-position, TMP exists in racemic form and is marketed under various trade names (Reynolds, 1989). The properties of TMP differ appreciably in many respects from those of IMI or DMI and from those of other TCAs (Lapierre, 1989).

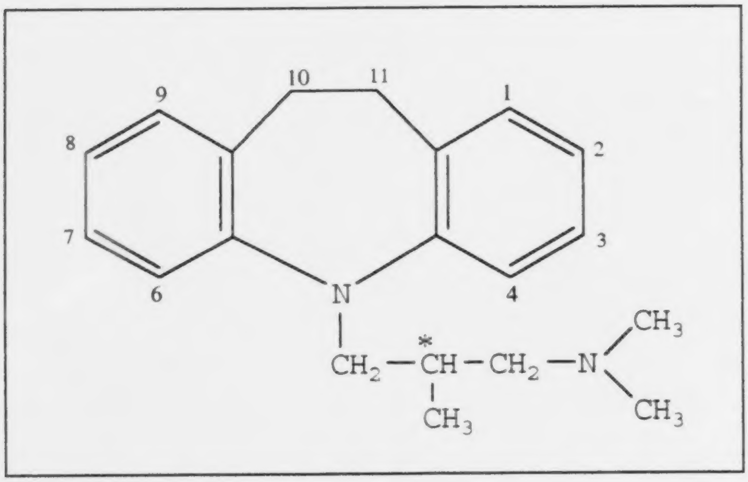

10
11
9
1
8
2
7
3
6
4
N
CH3
CH2
*
CH
CH2
N
CH3
CH3

Figure 1-1. Chemical structure of trimipramine (TMP).

* Denotes asymmetric center

1.1.1.2 Pharmacology

The pharmacology of the TCAs is complex and the precise mechanism of antidepressant action of these drugs is unclear, but they have been shown to block, in varying degrees, the reuptake of various neurotransmitters at the neuronal membrane (Alpers and Himwich, 1969; Axelrod, 1971). The postsynaptic effects of NE and 5HT are thus potentiated. In addition, most TCAs (*e.g.* IMI, TMP, AMI etc.) exhibit strong anticholinergic activity. The antidepressant activity of these drugs may be related to any or all of these effects, although the effects on NE and 5HT are considered to be the most important (McEvoy *et al.*, 1991). However, in recent years, animal studies have produced evidence that chronic administration of antidepressants can alter the sensitivity of membrane receptors of neurotransmitters in the brain. These findings, coupled with the introduction of newer ADs that have no apparent effect on either 5HT or NE, have led to revisions of the biogenic amine hypotheses (Prien and Blaine, 1984). The newer hypotheses link depression (and the action of ADs) not only to neurotransmitter uptake but to changes in the sensitivity of the pre- and post-synaptic neurotransmitter receptors at critical brain loci. Thus, depression is linked with supersensitivity of these receptors and the ADs work to reduce this phenomenon. In contrast, mania is associated with subsensitized (desensitized) receptors and anti-manic drugs, *e.g.* lithium, presumably increase the sensitivity of these receptors (Richelson, 1984).

TMP is a weak inhibitor of NE reuptake, but it inhibits 5HT reuptake and exhibits some dopamine receptor blocking activity as well as MAO inhibition (Lapierre, 1989). In addition to being an effective antidepressant, TMP is believed by some to be a potent histamine H_2 receptor antagonist (Alvarez *et al.*, 1986) and effective in the treatment of duodenal and gastric ulcer (MacKay *et al.*, 1984). TMP also significantly reduces joint pain and tenderness in rheumatoid arthritic patients (MacFarlane *et al.*, 1986). These diverse pharmacological properties of TMP may be associated with some of its many metabolites.

1.1.1.3 Side effects and toxicity

Minor adverse effects associated with tertiary amine TCAs generally reflect the drugs' anticholinergic and CNS activities. Serious reactions requiring discontinuation of therapy are relatively rare. The anticholinergic effects include dry mouth, blurred vision, constipation and urinary retention (Robinson, 1984; Horwell, 1985). Adverse CNS and neuromuscular effects occur frequently. Drowsiness is the most frequent adverse reaction to TCAs; weakness, lethargy, and fatigue are also common. In an attempt to reduce these side effects secondary amine TCAs were developed (*e.g.* DMI, NT), but these compounds are more stimulating and can aggravate anxiety and tension (Hordern, 1965).

The side effects of TMP are similar to the profiles of other tertiary amine TCAs, with some variations more characteristic of TMP. Sedation is a common side effect. Anticholinergic effects are also present with TMP; dry mouth is the most commonly reported symptom. Other side effects include blurred vision, constipation and urinary retention (Lapierre, 1989).

1.1.2 Iprindole (IPR)

1.1.2.1 Physicochemical properties

IPR is one of the so-called second generation ADs, marketed by Wyeth (U.K.) under the brand name Prondol®. It differs structurally from most other TCAs by being an indole derivative with an eight membered saturated third ring instead of an aromatic ring. The chemical structure of IPR, 5-(3-dimethylaminopropyl)-6,7,8,9,10,11-hexahydro-5H-cyclo-oct[b]indole, is shown in figure 1-2. IPR was introduced clinically in 1967 but has not attained the popularity of many other ADs despite the claim that it is as effective as, and is much less toxic than, other ADs (Cassidy and Henry, 1987). This might be due to a lack of well documented superiority to the other TCAs and because of clinicians' familiarity with the classic tricyclic drugs. A recommended daily dosage is 90-180 mg. As with other TCAs, onset of antidepressant activity is delayed for 2-3 weeks.

IPR hydrochloride is a white or nearly white odorless powder, soluble in water, chloroform and alcohol. It has a melting range of 142-147°C (Wyeth, 1972).

1.1.2.2 *Pharmacology*

The mechanism of antidepressant action of IPR is still unclear; it is not an inhibitor of NE reuptake (Lahti and Maickel, 1971), but appears to enhance adrenoreceptor activity by an unknown mechanism (Gluckman and Baum, 1969). Two of the rings of the standard tricyclic configuration are replaced by an indole moiety which renders IPR nearly devoid of MAO activity or the ability to block uptake of biogenic amines (Zis and Goodmin, 1979). Green *et al.* (1989) demonstrated that IPR provided partial protection of mouse brain MAO from the irreversible MAO inhibitor, phenelzine (PZN). The authors suggested that IPR reversibly inhibited MAO-A and MAO-B *in vivo*. Studies have indicated that IPR is a potent inhibitor of metabolic aromatic hydroxylation of various substrates (Freeman and Sulser, 1972; Sedlock et al., 1985; Mosnaim *et al.*, 1989). It was demonstrated that nonresponders to IPR improved with the addition of lithium (de Montigny, 1985).

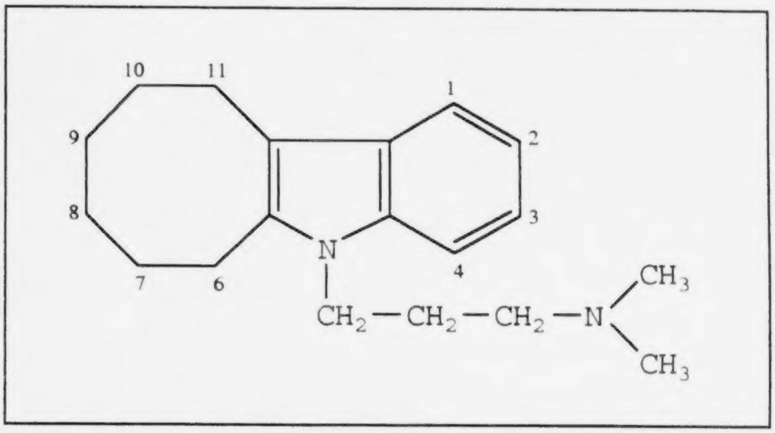

10
11
1
2
3
4
9
8
7
6
N
CH₂—CH₂—CH₂—N
CH₃
CH₃

Figure 1-2. Chemical structure of iprindole (IPR).

1.1.2.3 *Side effects and toxicity*

Gluckman and Baum (1969) reported that with IPR the incidence of side effects, especially anticholinergic or antihistaminic effects, is lower than could normally be expected with the iminodibenzyl derivatives. However, several reports of jaundice following treatment with IPR were observed, although it was mild and patients recovered in a matter of days or weeks after the cessation of treatment (Wyeth, 1972).

1.2 Metabolism

Metabolism is defined as the chemical modification of endogenous substances (*e.g.* fatty acids, steroids, prostaglandins, neurochemicals) or exogenous substrates (drugs and other xenobiotics) in a biologic environment. It is enzymatically controlled; oxidases, reductases, esterases, and enzymes involved in conjugation reactions are of particular importance. Metabolism plays a central role in the elimination of drugs and other xenobiotics from the body and has an important bearing on the therapeutic and toxic properties of these substrates.

As early as 1859, Buchheim, a German scientist and the very founder of modern pharmacology, made the following statement:

> *"In order to understand the actions of drugs it is an absolute necessity to have knowledge of the transformations they undergo in the body. It is obvious that we must not judge drugs according to the form and amount administered, but rather according to the form and amount which actually is eliciting the action (Conti and Bickel, 1977).*

What Buchheim anticipated became one of the chief reasons for the pharmacologists' interest in drug metabolism and for its amazing development during the 20th century. Benzoic acid was the first compound whose metabolic fate in mammals was studied in the 1840s (Conti and Bickel, 1977). The metabolic pathway involved was conjugation with glycine to form hippuric acid (Figure 1-3).

Latter in the 19th century metabolic oxidation, reduction, sulfate formation and glucuronic acid conjugation were discovered. With the advent of sophisticated analytical instruments and advancement of knowledge in the drug metabolism field scientists are now able to isolate, purify and characterize multiple forms of enzymes responsible for xenobiotic metabolism.

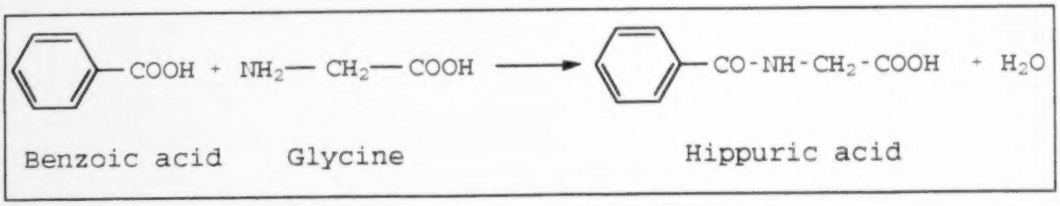


Figure 1-3. Metabolic formation of hippuric acid

If lipophilic drugs or xenobiotics were not metabolized to polar, water-soluble products that are readily excretable, they would remain indefinitely in the body eliciting their biological effects. Thus, drug metabolism reactions have been regarded as detoxification processes (Williams, 1959). However, it is not correct to assume that drug metabolism reactions are always detoxifying. Many drugs are biotransformed to pharmacologically active metabolites (Potter, 1981). In some cases, the parent compound (pro-drug) is inactive and must be converted to a pharmacologically active metabolite (Drayer, 1976 and Colvin, 1976). Indeed many toxic side effects (*e.g.* tissue necrosis, carcinogenicity, teratogenicity) of drugs and environmental contaminants can be directly attributable to the formation of chemically reactive metabolites that are highly detrimental to the body (Gillette, 1974). Drug metabolism reactions have been divided into two categories: phase I (functionalization) and phase II (conjugation) reactions (Williams, 1959; Testa *et al.*, 1978; Brosen, 1990). Drug elimination *via* metabolism is thus normally a multistep process involving both phase I and phase II reactions. Some drugs, including acetazolamide, barbital and penicillin G, are mainly excreted in unchanged form.

1.2.1 Phase I metabolic reactions

Metabolic drug oxidations are referred to as Phase I or functionalization reactions. The purpose of phase I reactions is to introduce a polar functional group (*e.g.* OH, COOH, NH_2) into the xenobiotic molecule, while phase II reactions attach polar and ionizable endogenous compounds, such as glucuronic acid, sulfate, or glycine, to form pharmacologically inactive and water-soluble conjugated products (Testa and Jenner, 1976). Drug metabolism occurs in various tissues, including gut wall, liver, lung, kidney and brain (Klippert and Noordhoek, 1983). However, it is clear that the liver is the principal site of drug metabolism and that the enzymes concerned are located primarily in the hepatic smooth endoplasmic reticulum (Remmer, 1970). Of the various phase I reactions, metabolic oxidation is by far the most common and important pathway in xenobiotic metabolism. The enzyme systems carrying out this biotransformation are referred to as mixed function oxidases (MFOs) or monooxygenases (Mason, 1965). This reaction requires both molecular oxygen and the reducing agent nicotinamide adenine dinucleotide phosphate (NADPH). During this oxidative process one atom of molecular oxygen is introduced into the substrate (*e.g.* RH) to form

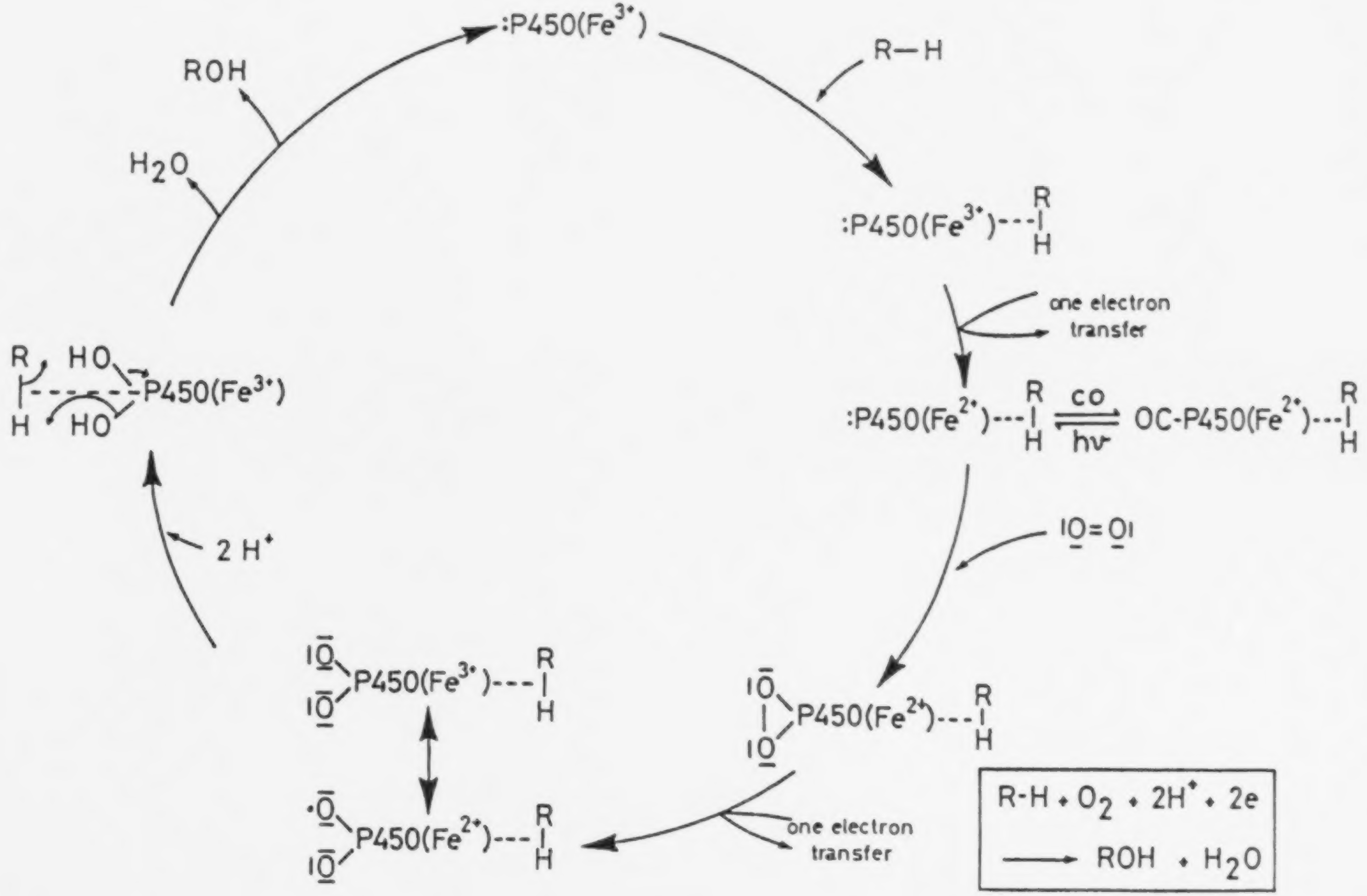

:P450(Fe^{3+})
R—H
:P450(Fe^{3+})---R-H
one electron transfer
:P450(Fe^{2+})---R-H
CO
hν
OC-P450(Fe^{2+})---R-H
O=O
O-O-P450(Fe^{2+})---R-H
one electron transfer
O-O-P450(Fe^{3+})---R-H
·O-O-P450(Fe^{2+})---R-H
2 H$^+$
R-H---HO-HO-P450(Fe^{3+})
H$_2$O
ROH
R-H + O$_2$ + 2H$^+$ + 2e ⟶ ROH + H$_2$O

Figure 1-4. Cytochrome P450-mediated carbon-oxidation cycle.

ROH, while the other oxygen atom is incorporated into water. Studies using $^{18}O_2$ have shown unequivocally that the oxygen atom in the metabolite is derived from air and not from water (Gibson and Skett, 1986). The mixed function oxidase system is actually made up of several components, the most important being an enzyme called cytochrome P450, which is responsible for transferring an oxygen atom to the substrate RH (Mannering, 1971). Other important components of this system include the NADPH-dependent cytochrome P450 reductase and a NADH-linked cytochrome b_5 (Testa and Jenner, 1976). NADPH cytochrome P450 reductase is responsible for the transfer of electrons from NADPH to cytochrome P450 (Lu and West, 1975) which then serves as the terminal monooxygenase for the metabolism of a wide variety of xenobiotics and endogenous substrates (Brosen, 1990). Figure 1-4 was devised by Dr. R.T. Coutts (unpublished data) and illustrates how cytochrome P450 is believed to be involved in metabolic C-oxidation.

Indeed it is recognized that the main function of phase I metabolism is to prepare the compound for phase II metabolism and not to prepare the drug for excretion (Gibson and Skett, 1986). Phase II metabolism is true detoxification and gives products that are generally water-soluble and easily excreted. Examples of phase I reactions (Testa and Jenner, 1976; Gibson and Skett, 1986) are listed in Table 1-1.

Table 1-1. Examples of phase I reactions

1. Oxidations involving MFO:
    - a) Oxidation of aromatic moieties
    - b) Oxidation of olefins
    - c) Oxidation of benzylic or allylic carbon atoms and carbon atoms alpha to carbonyl and imines
    - d) Oxidation at aliphatic and alicyclic carbon atoms
    - e) Oxidation involving carbon-heteroatom systems:
        - i) Carbon-nitrogen systems: include N-dealkylation, oxidative deamination, N-oxide formation, N-hydroxylation
        - ii) Carbon-oxygen systems: include O-dealkylation
        - iii) Carbon-sulphur systems: include S-dealkylation, S-oxidation
    - f) Oxidations of alcohols and aldehydes
2. Some metabolic oxidations are also catalyzed by enzymes other than MFO:
    - a) Alcohol dehydrogenase: this enzyme catalyzes the oxidation of many alcohols to their corresponding aldehydes.

b) Xanthine oxidase: metabolizes xanthine-containing drugs, *e.g.* caffeine, theophylline and purine analogs, to the corresponding uric acid derivative.

c) Amine oxidases: this group of enzymes is subdivided into two classes:

  i) Monoamine oxidase (MAO), which metabolizes endogenous catecholamines, *e.g.* 5-HT, dopamine, NE and dietary exogenous amines,*e.g.* tyramine (found in cheese).

  ii) Diamine oxidase, responsible for deaminating endogenous diamines, *e.g.* histamine.

d) Aromatases: responsible for converting xenobiotics containing a non-aromatic moiety to an aromatic moiety, *e.g.* metabolism of cyclohexanecarboxylic acid to benzoic acid; synthesis of female sex hormones.

3. Reductive reactions:

These reactions are catalyzed by enzymes localized in hepatic microsomes and require NADPH but are inhibited by oxygen. Examples of reductive reactions include:

a) Reduction of aldehydes and ketones to alcohols

b) Reduction of nitro and azo compounds to hydroxylamines and amines

c) Miscellaneous reductive reactions

4. Hydrolytic reactions:

Hydrolytic enzymes (hydrolases) are present in many organs, including liver, kidney, plasma and intestine. Intestinal microorganisms also play an important role in hydrolytic reactions (Testa and Jenner, 1976).

a) Hydrolysis of esters and amides: these reactions are catalyzed by arylesterases, carboxylesterases, acetylcholinesterases, pseudocholinesterases and liver amidases.

b) Hydrolysis of hydrazide and carbamate.

1.2.1.1 *The Cytochrome P450 isozyme systems*

Cytochrome P450, subsequently referred to simply as P450, is the collective term for a group of related enzymes which are responsible for the oxidation of endogenous substances, numerous drugs, and other xenobiotics (Guengerich, 1991; Brosen, 1990). These enzymes are located in the membranes of the endoplasmic reticulum of the liver. The presence of this enzyme in many other tissues (*e.g.* lung, kidney, intestine, skin, placenta, adrenal cortex) is a reflection that these tissues also have drug oxidizing capacity (Vanio and Hietanen, 1980; Shahi *et al.*, 1990). The discovery that P450s are, in fact, primary enzymes of drug metabolism derived in part from experiments which showed that drug metabolism rises and falls in parallel with changes in P450 levels (Nebert and Gonzalez, 1987). The liver contains multiple P450s, probably between 20 and 100, and these have variable and often overlapping reactivities toward various drug substrates (Gonzalez, 1990; Nebert and Gonzalez, 1987). The name cytochrome P450 is derived from the fact that the reduced form of this enzyme binds with carbon monoxide to form a complex that absorbs at 450 nm (Omura and Sato, 1964) and P stands for protein. The molecular weight ranges from 45,000 to 55,000 daltons (Gibson and Skett, 1986) and P450s differ by differences in their amino acid sequences (Brosen, 1990).

Each cytochrome P450 consists of a single protein (called apoprotein) and contains 1 heme group as the prosthetic group (the nonprotein component of a conjugated protein enzyme) (Gibson and Skett, 1986; Brosen, 1990). Each P450 is encoded by a separate gene. Thus P450s which are less than 36% similar in amino acid sequence belong to different families. Eight different P450 families are known in humans (Nebert *et al.*, 1989; Brosen, 1990). Drug metabolizing P450 isozymes belong to families I, II, III and IV, while the remaining P450s metabolize endogenous substrates and other foreign compounds (Brosen, 1990). Subfamilies are also known and are found only within family II. Each drug metabolizing P450 has wide substrate specificity and thus can catalyze the oxidation of several drugs. However, a drug may have a very high affinity for one particular P450 isozyme and that drug will be metabolized almost exclusively by this particular isozyme. P450s are difficult to purify. It is the rule, rather than the exception, that purified cytochrome preparations are often contaminated with other P450s.

The nomenclature of cytochrome P450 isozymes is based on a method developed by Nebert *et al.* (1989). In this system of nomenclature, the Roman numeral which follows P450 designates the family of the isozyme, a capitalized alphabetic letter which follows the Roman numeral indicates the subfamily and finally, the individual gene is designated by an Arabic numeral.

Table 1-2. *Human cytochrome P450 isozymes*

Human P450 isozymes that have been characterized at least to some extent are:

P450IA1, P450IA2, P450IIA3, P450IIB6, P450IIB7, P450IIB8, P450IIC9, P450IIC10, P450IID6, P450IID7, P450IID8, P450IIE1, P450IIF1, P450IIIA1, P450IIIA3, P450IIIA4, P450IIIA5, P450IVB1, P450XIA1, P450XVIIA1, P450XIXA1and P450XXIA2 (Gonzalez *et al.*, 1987; Nebert *et al.*, 1989; Brosen, 1990; Okey, 1990; Coutts, 1991a).

Table 1-3. *Animal cytochrome P450 isozymes*

Animal P450 isozymes known include:

P450IA1, P450IA2, P450IIA1, P450IIA2, P450IIA3, P450IIB1, P450IIB2, P450IIB3, P450IIB4, P450IIB5, P450IIB9, P450IIB10, P450IIC1, P450IIC2, P450IIC3, P450IIC4, P450IIC5, P450IIC6, P450IIC7, P450IIC11, P450IIC12, P450IIC13, P450IIC14, P450IIC15, P450IID1, P450IID2, P450IID3, P450IID4, P450IID5, P450IID6, P450IID9, P450IID10, P450IIE1P450IIE2, P450IIG1, P450IIH1, P450IIIA1, P450IIIA2, P450IIIA4, P450IIIA6, P450IVA1, P450IVA2, P450IVA3, P450IVA4, P450IVA5, P450IVA6, P450IVA7, P450IVB1, P450VIA1, P450XIA1, P450XIA2, P450XVIIA1, P450XIXA1 and P450XXIA1 and P450XXVIA1 (Gonzalez *et al.*, 1987; Nebert *et al.*, 1989; Brosen, 1990; Okey, 1990; Coutts, 1991a).

Characteristics and assay methods of some of the important isozymes are described below (Coutts, 1991a):

P450IA1

a. This is rat P450c. Acetaminophen binds to it.

b. Assay: 7-ethoxyresorufin O-deethylase or arylhydrocarbon hydroxylase activities.

c. Inducible by polycyclic aromatic compounds in human, rat, mouse and rabbit .

d. Only inducible P450 species located in human placenta.

e. Also expressed in rat, mouse, rabbit and trout.

P450IA2

a. Assay: acetanilide 4-hydroxylase, 7-ethoxyresorufin O-deethylase, estradiol 2-hydroxylase and phenacetin O-deethylase activities.

b. Inducible by polycyclic aromatics, *e.g.* 3-methyl cholanthrene, in human, rat, mouse and rabbit (Okey, 1990).

c. Also expressed in rat, mouse and rabbit.

P450IIC9

a. Assay: mephenytoin 4'-hydroxylase activity.

b. This isozyme exhibits polymorphism.

c. About 3% of Caucasians and over 20% of Japanese are poor metabolizers (PM) of mephenytoin.

P450IID6

a. By far the best characterized P450. Formerly known as P450dbl.

b. Assay: bufuralol 1'-hydroxylase and debrisoquine 4-hydroxylase activities.

c. Intact isozyme is absent or almost absent from the livers of poor metabolizer (PM) of debrisoquine, or it is present in a functionally altered form.

d. With some substrates of psychiatric interest, *e.g.* IMI, DMI and other substrates, *e.g.* antiarrhythmic drug, propafenone, P450IID6 becomes saturated at therapeutic doses. It is, therefore, of clinical significance to phenotype patients for P450IID6 activity prior to commencing treatment with tricyclic antidepressants, neuroleptics (*e.g.* perphenazine, thioridazine) and antiarrythmics.

e. Possible links between Parkinson's disease and cancer and PMs of debrisoquine have been reported.

f. Apparently P450IID6 cannot be induced (Eichelbaum *et al.*, 1986)

g. P450IID6 apparently has no known endogenous substrates. It metabolizes only basic substrates of which there are many examples, *i.e.* it has broad substrate specificity. All its substrates undergo metabolic oxidation at a site located 0.5-0.7 nm from the basic nitrogen atom. This isozyme is also detected in human brain.

h. Dark Agouti (DA) rats have five P450s (IID1-IID5) closely related in structure to P450IID6; one of these five isozymes may be identical to, or very closely related in structure to P450IID6. Gonzalez *et al.* (1987) have demonstrated that P450IID2 and P450IID6 are expressed in male Sprague-Dawley rat liver.

A drug may be identified as a potential substrate for a particular isozyme by determining its ability to act as a competitive inhibitor of the oxidation of a model drug in a human *in vitro* liver microsomal preparation. In the case of P450IID6, debrisoquine, sparteine, dextromethorphan, DMI, and bufuralol can be used as the model drug (Brosen, 1990). A drug that does not inhibit the oxidation of the model drug will not be a substrate for that particular isozyme. Conversely, a drug that is a competitive inhibitor is likely to be a substrate. If bufuralol is used as a model substrate for the detection of competitive inhibitors of P450IID6, or for the identification of PMs who lack P450IID6, as little as 5 mg of human liver is sufficient (Meyer *et al.*, 1990). The cytochrome P450IID6 isozyme has a wide substrate specificity *i.e.* it catalyzes the ring hydroxylation of many aromatic basic drugs of clinical importance (Jacqz *et al.*, 1986; Kalow, 1986; Brosen, 1990; Brosen et al., 1991). It is expected that other human drug metabolizing P450s will be similarly characterized in future.

1.2.2 Phase II metabolic reactions

Phase II metabolism involves a diverse group of enzymes acting on diverse types of compounds, generally leading to

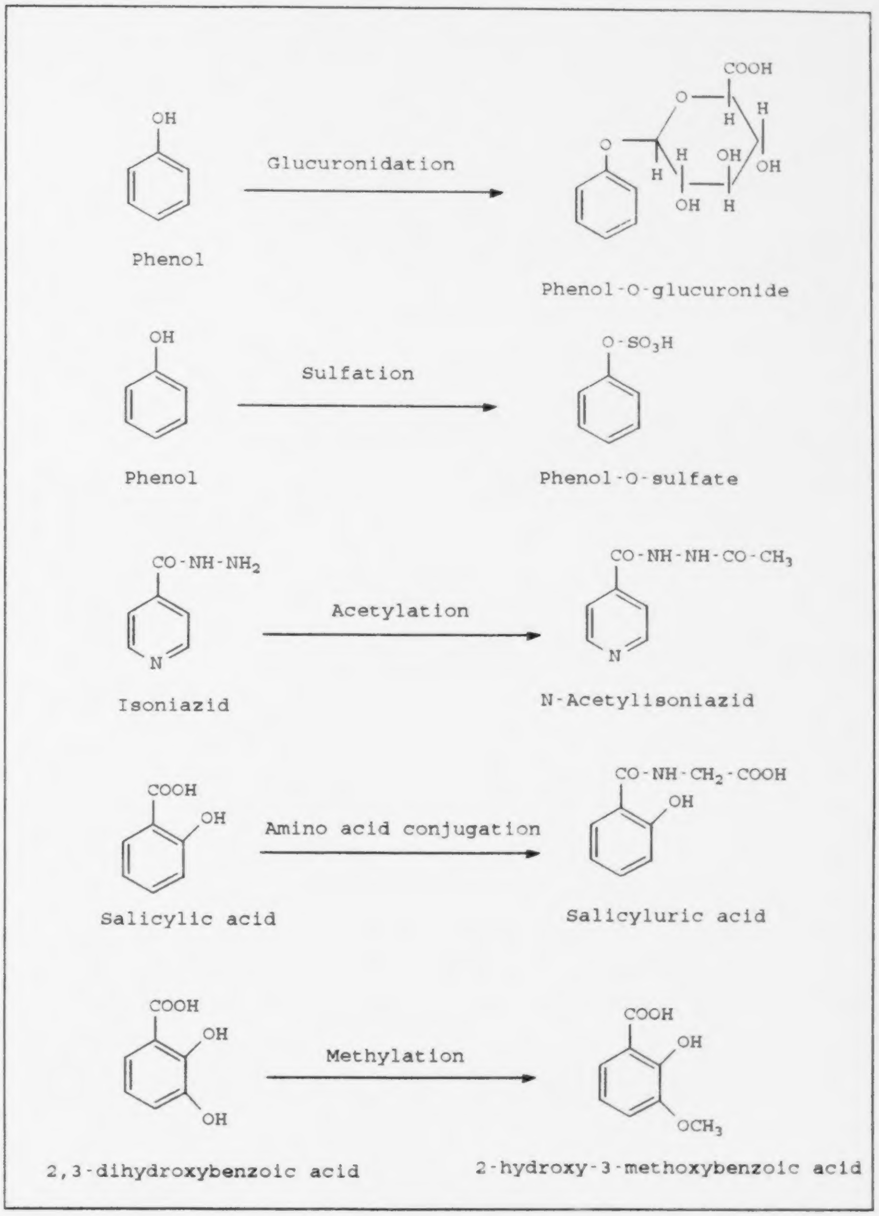

OH
Glucuronidation
COOH
O
H
H
OH
OH
OH
H
Phenol
Phenol-O-glucuronide
OH
Sulfation
O-SO$_3$H
Phenol
Phenol-O-sulfate
CO-NH-NH$_2$
Acetylation
CO-NH-NH-CO-CH$_3$
N
Isoniazid
N-Acetylisoniazid
COOH
OH
Amino acid conjugation
CO-NH-CH$_2$-COOH
OH
Salicylic acid
Salicyluric acid
COOH
OH
OH
Methylation
COOH
OH
OCH$_3$
2,3-dihydroxybenzoic acid
2-hydroxy-3-methoxybenzoic acid

Figure 1-5. Schematic representation of some phase II metabolic reactions.

water-soluble products which can be excreted in bile or urine. A distinguishing feature of most phase II reactions is that the conjugating group (glucuronic acid, sulfate, methyl or acetyl) is initially activated in the form of a coenzyme before transfer or attachment of the group is made to the accepting substrate by the appropriate transferase enzyme. In other cases, such as glycine and glutamine conjugation, the substrate is initially activated. Conjugates are predominantly inactive pharmacologically, although there are examples of conjugates which do retain pharmacological activity (Yoshimura H. *et al.*, 1973; Oguri K. *et al.*, 1984). Examples of phase II reactions (Gibson and Skett, 1986) are listed in Table 1-4.

Table 1-4. Examples of phase II reactions

Reaction	**Functional group**
a) Glucuronidation	$-OH, -COOH, -NH_2, R-N(CH_3)_2$
b) Sulfation	$-NH_2, -OH$
c) Methylation	$-OH, -NH_2$
d) Acetylation	$-NH_2, -OH$
e) Amino acid conjugation	$-COOH$
f) Glutathione conjugation	Epoxide, Organic halide

1.2.3 Metabolism of tricyclic antidepressants (TCAs)

The major factor responsible for observed inter-individual and inter-species pharmacokinetic variations is considered to be the drug metabolism process, whereas absorption, distribution and excretion show less pronounced variation (Brodie, 1964). The steady-state plasma levels of TCAs show large individual variation (Hammer and Sjoqvist, 1967). Of the two factors determining steady-state plasma levels, *i.e.*, distribution and elimination, the latter seems to be the more important (Wagner *et al.*, 1965). Variations in elimination capacity must, therefore, be considered as important factors determining the effect of TCAs.

The TCAs are highly lipophilic and therefore subject to multiple biotransformation steps yielding polar metabolites that can be readily excreted in the urine or bile (Potter, 1981; Rudorfer and Potter, 1987). The major site of this drug metabolism is the liver, where TCAs undergo mainly demethylation and hydroxylation followed by glucuronide conjugation (Gram, 1974; Potter and Manji, 1990; Devane, 1986). The oxidative reactions are usually the rate-limiting ones and are subject to stimulation or inhibition by exogenous agents (Rudorfer and Potter, 1987). Overall, renal clearance accounts for elimination of less than 5% of administered doses as unchanged drug (Sutfin *et al.*, 1984; Sjoqvist *et al.*, 1969). TCAs that have received the most attention in drug metabolism and pharmacokinetic studies are

IMI, DMI, AMI and NT. The metabolism and pharmacokinetic properties of PROT, CMI and the newer antidepressants, DOX, trazodone, mianserin, bupropion, maprolitine and amoxapine, have been less thoroughly investigated (Rudorfer and Potter, 1985). TMP and IPR are two antidepressants whose metabolic and pharmacokinetic properties have been virtually ignored.

Amitriptyline (AMI):

The main metabolic pathways of AMI are N-demethylation and alicyclic oxidation at C_{10} (Rollins *et al.*, 1980). Therefore the major metabolites are 10-OH-NT and 10-OH-AMI. Bertilsson *et al.* (1981) reported that 3-5% of AMI is excreted in urine as 10-OH-AMI and about 40% is excreted as 10-OH-NT. In another study (Vandel *et al.*, 1982) it was found that 10-OH-NT accounted for 55% of an oral dose of AMI. Human urine metabolites of AMI were quantified in three patients by Prox and Breyer-Pfaff (1987) who showed that the total recoveries were only 28.6-60.2% of the administered dose. Rudorfer and Potter (1985) have provided a possible explanation for such low recoveries. They stated that both TCAs and their metabolites undergo enterohepatic circulation, and failure to account for drug excreted *via* bile and feces will result in apparently low recoveries (60-70%) of administered TCAs.

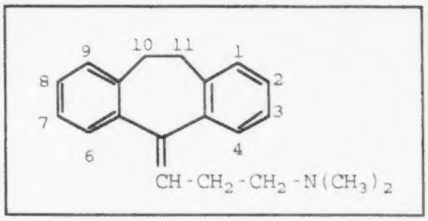


Figure 1-6. Structure of amitriptyline (AMI)

The metabolism of AMI in rats, and the identification of metabolites in rat bile have recently been reported by Baier-Weber *et al.* (1988). Glucuronides isolated include those of 2-OH-AMI, 10-OH-AMI, 2-OH-3-OMe, 10,11-$(OH)_2$-AMI, and some N-demethylated analogs of these metabolites.

Nortriptyline (NT):

The major metabolic pathway identified for NT is alicyclic oxidation at C_{10}. Rudorfer and Potter (1985) found that, about 50% of an orally administered dose of NT is excreted in the urine as 10-OH-NT. This conclusion is in agreement with the observations made by Alexanderson and Bogra (1973) that 62% of a dose of NT could be accounted for by urinary metabolites after both single and multiple doses, and that, of the total urinary metabolites, 80-82% was 10-OH-NT, both conjugated and unconjugated; 1% was desmethylnortriptyline (DNT) and 16-18% was 10-OH-DNT, mainly conjugated.

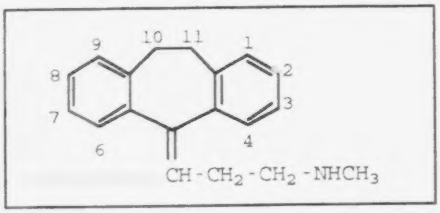


Figure 1-7. Structure of nortriptyline (NT).

Imipramine (IMI):

IMI is the most thoroughly investigated TCA. IMI is mainly eliminated by demethylation to the active metabolite desipramine (DMI) and to a lesser extent by aromatic hydroxylation to 2-OH-IMI. DMI is further metabolized by aromatic hydroxylation to 2-OH-DMI (Sallee and Pollock, 1990). Quantitatively, hydroxylation is the most important intermediate metabolic pathway facilitating the clearance of IMI (Rubinstein *et al.*, 1983). The greater plasma elimination half-life for DMI compared to IMI could be explained by lower hydroxylation rates for DMI in microsomal preparations (Kruger *et al.*, 1986). Minor metabolic pathways for IMI are dealkylation of the entire side chain to form iminodibenzyl (IDB) or further demethylation of DMI to didesmethylimipramine (DDMI) and subsequent 10-hydroxylation (Sallee and pollock, 1990).

The demethylation and aromatic hydroxylation of IMI are carried out by at least 2 different cytochrome P450 isozymes (von Bahr *et al.*, 1985 and Brosen *et al.*, 1986a,b). The aromatic hydroxylation of IMI and DMI is catalyzed by cytochrome P450IID6 isozyme and as such inter-individual differences exist in their metabolism (Sallee and Pollock, 1990).

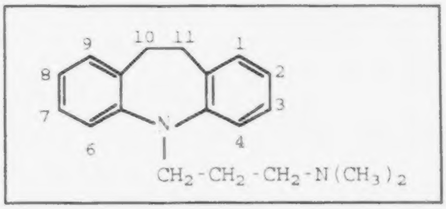


Figure 1-8. Structure of imipramine (IMI)

Less than 5% of an oral dose of IMI is excreted unchanged in the urine (Sjoqvist *et al.*, 1969). The most important IMI metabolites (free and conjugated) are 2-OH-DMI (40-50%), 2-OH-IMI (15-25%) and 2-OH-IDB (15%) (Crammer *et al.*, 1969; Rudorfer and Potter, 1985; Potter *et al.*, 1982; Potter and Manji, 1990). IMI-N-oxide, IDB and 2-OH-IDB are formed in humans in significant amounts; IMI-N-oxide is metabolically reconverted into IMI, *i.e.* it is a "predrug" of IMI. IDB and 2-OH-IDB are of little clinical significance (Potter and Calil, 1981). Gram et al., (1983) conducted a study on 24 patients receiving DMI and demonstrated that in some of these patients, especially those who were poor hydroxylators of IMI and DMI, plasma levels DDMI were appreciable, often exceeding those of 2-OH-IMI. In 13 of the 24 patients, however, DDMI could not be detected in plasma.

Clomipramine (CMI):

CMI is extensively metabolized, with only 1-3% of a dose excreted unchanged. Demethylation to desmethylclomipramine (DCMI) is the major metabolic pathway but hydroxylation and N-oxidation are also important (McTavish and Benfield, 1990; Gex-Fabry M *et al.*, 1990). The mean concentrations of major metabolites of CMI in plasma at steady-state after a po dose (200 mg CMI per day) include DCMI (313.0 ng/ml), 8-OH-DCMI (152.7 ng/ml), free CMI (147.5 ng/ml) and 8-OH-CMI (56.1 ng/ml) (Linnoila et al., 1982). The plasma DCMI:CMI ratio is 2 to 3 times higher after oral CMI administration compared with parenteral administration, suggesting that the drug is subject to first-pass hepatic elimination (Nagy and Johansson, 1977). Of a single oral CMI dose 50-60% was accounted for in urine and about 30% in faeces after 14 days (Faigle and Dieterle, 1973). Mean plasma elimination half-lives of CMI were 20-26h in healthy volunteers (Nagy and Johansson, 1977). The elimination half-life of DCMI in depressed patients after oral administration of CMI is 50h, considerably longer than that of the parent drug (Perel *et al.*, 1986).

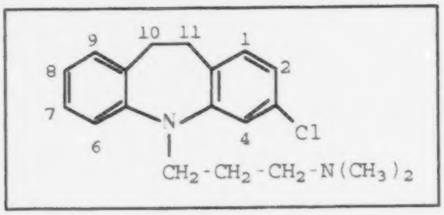


Figure 1-9. Structure of clomipramine (CMI)

Protriptyline (PROT):

The metabolism of PROT has not been extensively studied. Sisenwine *et al.* (1970) demonstrated that this compound is metabolized by 10-hydroxylation followed by conjugation in three species (pig, dog and man), but demethylation to the primary amine occurred only in the dog.

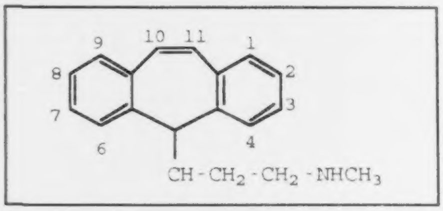


Figure 1-10. Structure of protriptyline (PROT)

Thus PROT, a secondary N-methylamine, undergoes very little N-demethylation in man. In contrast, fenfluramine, a secondary N-ethylamine, is extensively N-deethylated to norfenfluramine in man (Beckett and Brookes, 1967). PROT's first-pass hepatic clearance is only about 10-25% (Zeigler *et al.*, 1978).

Trimipramine (TMP):

TMP, like other TCAs, is rapidly absorbed from the GI tract and peak plasma levels follow a single oral dose by 2 to 4h (Lapierre, 1989; Koppel and Tenczer, 1988). Its first-pass hepatic clearance is high and its steady-state is related to dose. The relationship of plasma concentration to therapeutic response is not yet well established. The elimination half-life of 24h for TMP is somewhat shorter than for most TCAs (Abernethy *et al.*, 1984).

The metabolism of TMP in dog and rabbit (Populaire *et al.*, 1970) and in man (Abernethy *et al.*, 1984; Suckow *et al.*, 1984; Koppel and Tenczer, 1988; Cowan *et al.*, 1988; Maurer, 1989) has been reported only recently. In the study reported by Maurer (1989), urine samples from 3 humans who had received 100 mg TMP and 2 urine samples from overdose victims were invesigated. Besides unchanged TMP, 15 metabolites were identified. Metabolites included 2-OH-TMP, norTMP, 2-OH-norTMP, iminodibenzyl (IDB), 2-OH-IDB, 2,3-di-OH-IDB, 2-OH-x-OCH_3-IDB, 2,3-di-OH-TMP, 2,3-di-OH-norTMP, didesmethyl-TMP (DDTMP), 2-OH-DDTMP, 2,3-di-OH-DDTMP, 2-OH-x-OCH_3-TMP, 2-OH-x-OCH_3-norTMP, 2-OH-x-OCH_3-DDTMP. It is interesting that no C_{10} or C_{11} oxidation was observed. The hydroxylated metabolites are excreted mainly as glucuronide conjugates. Suckow *et al.* (1984) identified some patients as poor N-demethylators of TMP. TMP in large overdose is also readily demethylated in man, and fatalities following its ingestion have been documented. Whole blood levels of TMP and norTMP were 400 and 1300 ng/ml, respectively in one instance (Meatherall *et al.*, 1983), and 4800 and 2100 ng/ml, respectively, in another (Fraser *et al.*, 1987).

The pharmacological effects of TMP metabolites have not been well documented. As DMI, the major metabolite of the structural analog IMI, is pharmacologically active, it seems to be likely that at least norTMP will also be active (Koppel and Tenczer, 1988). No metabolic study for TMP has yet been reported in the rat.

Iprindole (IPR):

Although IPR has been on the market for more than 20 years, little is known about IPR's metabolic and pharmacokinetic properties. Surprisingly, no comprehensive metabolism studies have yet been reported for IPR in any species. However, a preliminary study on the metabolism of IPR in various mammals including rat (species not identified) has been published (Sisenwine *et al.*, 1979). The metabolic pathways identified were N-demethylation, alicyclic oxidation and a combination of both pathways. Caille *et al.* (1982) have found that the plasma elimination half-life of IPR in human is 52.5h.

1.2.3.1 Active metabolites of TCAs

Most of the currently available TCAs are biotransformed into one or more active metabolites in humans and/or animals(Bertilsson *et al.*, 1979 and 1981; Potter *et al.*, 1982; Rudorfer and Potter, 1985). In some cases these metabolites attain blood and/or brain levels within the same range as, or even higher than, those of the parent drug. The formation of metabolites with their own biological activity in addition to that of the parent compound may result in a complex situation where different chemical entities participate in the final effects. They may act by similar mechanisms, by different mechanisms or even antagonistically. These active metabolites may not only contribute to the therapeutic outcome of a drug but perhaps also to the toxicity of a drug. Therefore, drug metabolism studies are essential for an understanding of the mechanism of action of TCAs, and for extrapolating pharmacological and toxicological findings from animals to humans (Caccia and Garattini, 1990).

N-Demethylation and hydroxylation of TCAs produce active metabolites (Bertilsson *et al.*, 1981). The pharmacological profiles of IMI, DMI and their 2-hydroxy derivatives, AMI, NT and their 10-hydroxy derivatives and CMI and DCMI have been well investigated (Bertilsson *et al.*, 1979 and 1981; Potter *et al.*, 1982; Rudorfer and Potter, 1985). Tertiary amine TCAs (*e.g.* IMI, AMI) are more potent inhibitors of 5-HT uptake, whereas secondary amine TCAs (*e.g.* DMI, NT) are relatively specific inhibitors of NE uptake (Maas, 1975; Randrup and Braestrup, 1977; Potter and Manji, 1990). Furthermore, in classifying TCAs according to potency on different receptors, it has been established that tertiary and secondary amines differ markedly in the extent of their interaction with histaminic, muscarinic and α-adrenergic receptors (Richelson and Nelson, 1984). Tertiary amines are consistently the most potent in terms of blocking histaminic, muscarinic and α-adrenergic receptors. DMI and NT were developed as antidepressants in their own right. What is less often appreciated is that tertiary amines are extensively metabolized by N-demethylation to secondary amines, and the plasma concentrations of secondary amine metabolite can exceed those of the tertiary amine drug resulting in extensive NE uptake inhibition (Bertilsson *et al.*, 1974). Furthermore, in one clinical study (Narasimhachari *et al.*, 1982) it was found that 15% of a group of patients treated with therapeutic doses of DMI had measurable amounts of IMI in their blood, presumably resulting from direct methylation of the parent drug DMI. A case report of a fatal NT overdose (2 g) also provided evidence of *in vivo* methylation (Rudorfer and Robins, 1985). Thus, no TCAs can be considered to be selective uptake inhibitors of 5HT following chronic oral administration. In contrast, the newer ADs, including fluoxetine, fluvoxamine and citalopram and their active metabolites are all selective 5HT reuptake inhibitors (Potter and Manji, 1990).

The hydroxylated metabolites of the TCAs (2-OH-IMI, 2-OH-DMI, 8-OH-CMI, 8-OH-DCMI, 10-AMI and 10-NT) have a spectrum of pharmacological activity similar to that of their respective tertiary or secondary amine parent compounds; all are virtually equipotent with the corresponding parent drugs in animal studies for antidepressant activity (Bertilsson *et al.*, 1981; Potter and Calil, 1981; Bertilsson *et al.*, 1979;

Potter *et al.*, 1979). Thus, there is a probability that 2- and 10-hydroxy TMP and IPR metabolites could also be pharmacologically active. Nagy and Hansen (1978) demonstrated that the activity of IMI-N-oxide might be of biologic significance. Very little is known about the didemethylated metabolites of TCAs and it would be of interest to determine the pharmacological properties of these minor metabolites (Bertilsson *et al.*, 1979).

Thus, according to Potter (1981), in terms of biologic activity, active forms of TCAs can be divided into two broad classes: tertiary and secondary amines. In each class there are two active forms with similar potencies: the 'parent' and the hydroxylated form. Pharmacological profiles of other forms have not been convincingly demonstrated.

1.3 Analysis of TCAs

Several analytical methods have been developed to monitor TCAs and their metabolites in biological fluids. The low concentrations of these drugs found in biological fluids and tissues presented formidable technical difficulties during the early years. Many attempts were made to develop suitable methods involving spectrophotometry, spectrofluorometry, thin-layer chromatography, radioimmunoassay and isotope derivative analysis. Contemporary methods of analysis for TCAs include gas chromatography with nitrogen-specific detection, high-performance liquid chromatography and radioimmunoassay. Mass fragmentographic methods are generally considered to be the most reliable and specific, but these techniques are expensive. Even with these advances, the TCAs still remain some of the most difficult drugs to analyze reliably (Scoggins *et al.*, 1980a and 1980b). However, because of the ease of operation, sensitivity and low cost, gas chromatography with nitrogen detection and high pressure liquid chromatography with ultraviolet detection are presently the methods of choice in the analysis of TCAs (Glassman *et al.*, 1985).

1.4 Enzyme induction and inhibition

Enzyme induction by drugs and other xenobiotics was discovered by virtue of the profound effects that induction can have on pharmacologic responses (Conney, 1967). For example, phenobarbital administered chronically to rats gradually reduced its own sedative effect by enhancing barbiturate metabolism and thereby clearance (Remmer and Merker, 1963). Common inducers include phenobarbital, 3-methylcholanthrene (3-MC), and ethanol (Okey, 1990). However, not all P450 isozymes are inducible. P450IIC7, P450IIC13 (Bandiera *et al.*, 1986) and P450IID6 (Eichelbaum *et al.*, 1986) are relatively refractory to induction by all common classes of P450 inducers.

P450 induction by phenobarbital in the liver is accompanied by a substantial increase in the content of smooth endoplasmic reticulum within the liver cells and by an increase in liver weight (Remmer and Merker, 1963). However, such morphologic changes are far less prominent with other inducers. Increased catalytic activity of P450-mediated monooxygenases is primarily the consequence of increased levels of P450 apoproteins and decreased degradation (Okey, 1990; Testa and Jenner, 1976). In turn, specific P450 isozyme levels are elevated predominantly as the consequence of increased gene transcription (Nebert and Gonzalez, 1987).

Inhibition of the P450-mediated MFO system has been the subject of many reviews (Boobis *et al.*, 1990; Murray, 1987; Testa and Jenner, 1981; Netter, 1980). This inhibition of drug metabolism by drugs or xenobiotics can take place in several ways including reversible inhibition, destruction of pre-existing enzymes, inhibition of enzyme synthesis or by complexing and thus inactivating the drug metabolizing enzyme (Gibson and Skett, 1986). The coadministration of drugs sometimes results in competitive, active site inhibitory interactions and may result in impaired drug elimination. A large number of drugs have been shown to inhibit the metabolism of other drugs both *in vivo* and *in vitro*. The onset of inhibition is usually rapid following a single dose of the inhibitory compound (Kato *et al.*, 1964) and from this point of view interactions involving inhibitors of drug metabolism are probably of greater significance than those involving enzyme induction (Testa and Jenner, 1976). Perhaps the most potent reversible MFO inhibitors are the nitrogen heterocycles (Murray, 1987). This class of compounds includes the imidazoles and quinolines, and these ring systems are present in a large number of therapeutic agents. An imidazole ring system is present in antifungal agents (*e.g.* ketoconazole, miconazole) and H_2-receptor antagonists (*e.g.* cimetidine), while a quinoline ring is present in antimalarial drugs like chloroquine, primaquine, quinine and in antiarrhythmic agents such as quinidine.

Quinidine (QND) is a potent competitive inhibitor of the P450IID6 isozyme, which has a broad substrate specificity (Otton *et al.*, 1984). Thus QND strongly inhibits the *in vivo* oxidative metabolism of sparteine (Brinn *et al.*, 1986), debrisoquine (Ayesh *et al.*, 1991; Brosen *et al.*, 1987), IMI (Brosen and Gram, 1989b), DMI (Ayesh *et al.*, 1991; Brosen and Gram, 1989b; Steiner *et al.*, 1988) metoprolol (Leeman *et al.*, 1986), amphetamine (Moody *et al.*, 1990), NT (Ayesh *et al.*, 1991) and all other drugs whose biotransformation is catalyzed by the P450IID6 isozyme. QND binds strongly to P450IID6, but is not itself metabolized by it (Guengerich *et al.*, 1986). QND inhibits the aromatic hydroxylation of IMI and DMI but not the demethylation of IMI (Brosen and Gram, 1989b). At concentrations of quinidine at which the P450IID6 function is abolished, the drug has virtually no effect on other cytochromes P450 (Gut *et al.*, 1986; Inaba *et al.*, 1985). The result of its use prior to, or concomitantly with, a drug that is metabolized by the P450IID6 isozyme is that the drug's metabolism is inhibited and known extensive metabolizers behave like poor metabolizers (Brosen, 1990). Many drug/drug interactions involving QND are of clinical significance since inhibition of drug metabolism could lead to drug accumulation and adverse effects (Brosen and Gram, 1989a).

Quinine (QNN), the diastereoisomer of QND, is also a P450 isozyme inhibitor, but its actions are different from those of quinidine. QND, but not QNN, inhibits the *in vivo* metabolism of methoxyphenamine (MP) in man (Muralidharan *et al.*, 1989a). In extensive metabolizers, prior administration of QND caused a significant decrease in % dose of MP excreted as O-demethyl-MP and 5-OH-MP. Similar results have been obtained with Lewis and Dark Agouti rats (Muralidharan *et al.*, 1989b). In this latter study, O-demethylation and aromatic hydroxylation of MP, which are known to involve P450IID6, were both significantly inhibited by QND in both rat strains, while N-demethylation, which does not involve P450IID6, was not significantly inhibited in either rat strain. Steiner *et al.* (1988) have demonstrated that both QND and QNN decreased the excretion of 2-OH-DMI in healthy male volunteers, but the effect of QND was much stronger than that of QNN.

Kobayashi *et al.* (1989) have found that in human liver microsomes QNN is 60 times less potent an inhibitor of debrisoquine 4-hydroxylase activity than is QND. In contrast, in rat liver microsomes QNN is approximately 50 times more potent than QND. These authors claimed that both compounds act as competitive inhibitors of P450IID6 isozyme. This study also revealed the danger of extrapolating findings from animal studies to humans.

1.5 Polymorphism in drug oxidation and phenotyping

Interest in polymorphic drug oxidation has grown rapidly over the past few years. There is now increasing evidence that links the expression, or lack thereof, of specific P450 isozyme(s) with altered metabolism, response and toxicity to certain drugs and to disease (Ayesh *et al.*, 1984; Kaisery *et al.*, 1987; Shahi *et al.*, 1990). As a result of these associations, interest has grown in characterizing individuals within the population with respect to the expression of certain P450s and further defining the clinical implications associated with their expression (Kalow, 1986; Jacqz *et al.*, 1986; Meyer *et al.*, 1990). It is now well recognized that wide inter-individual variations in the extent of elimination can be anticipated for drugs which are predominantly eliminated by metabolism. The capacity of an individual to metabolize drugs is dependent upon physiological, environmental and genetic factors. Numerous studies have investigated the impact of environmental factors (Dollery *et al.*, 1979; Vessel, 1973) but less information is available concerning genetic regulation. These factors vary from subject to subject. The result is that marked inter-individual differences in responses to drugs are encountered.

Some metabolic reactions are dominantly under genetic control and exhibit polymorphism in the population *i.e.* the enzymes involved exist in several distinct forms due to genetic differences. Pharmacogenetics is concerned with the contribution of genetic factors to the variability of response to drugs. The first reports of the extent to which genetic variations contribute to inter-individual variations were related to acetylation of sulfonamides and hydrazine drugs (*e.g.* isoniazid, phenelzine, hydralazine) and the hydrolysis of succinylcholine and paraxon. The distribution of fast and slow acetylators was almost equal (Hughes *et al.*, 1954).

Hydrolysis and acetylations, however, are relatively unimportant metabolic reactions. For this reason, very little progress was made in polymorphic drug metabolism for some years. This is surprising in view of the large number of metabolic oxidation reactions that were known at that time to be catalyzed by cytochrome P450 isozymes. It was not until investigators started to study the excretion of drugs, especially in urine and bile, that significant differences in drug metabolism became noticeable (Idle and Smith, 1979). Thus, Shahidi (1968) reported the inability of two sisters to deethylate phenacetin; these sisters were more sensitive to the methemoglobinemia-producing effects of the drug (because the alternative metabolic reaction to *p*-phenetidine occurred). Latter on the hydroxylation of phenytoin was observed to be defective in three different families, but reasons for these defects were not offered (Kutt, 1971). However, it was not until 1975 that genetic polymorphisms of oxidative metabolism were reported, with the simultaneous publication of two genetic defects associated with sparteine (an oxytocic agent) oxidation in a German population (Eichelbaum *et al.*, 1979) and debrisoquine (an antihypertensive drug) hydroxylation in an English population (Mahgoub *et al.*, 1977). An individual with deficient metabolic capacity for a certain drug is called a PM as compared to the normal extensive metabolizer (EM).

1.5.1 Sparteine and debrisoquine

Sparteine/debrisoquine oxidation polymorphism is a reflection of differences in the activity of P450IID6 isozyme (Brosen and Gram, 1989a). Recent studies strongly suggest that P450IID6 is absent altogether in PMs, but other P450s may contribute to a minor extent to the oxidation of at least some compounds that are mainly dependent on P450IID6 for elimination (Boobis *et al.*, 1983; Zanger *et al.*, 1988; Gonzalez *et al.*, 1988). Cytochrome P450IID6 isozyme has a broad substrate specificity (Brosen, 1990). The sparteine /debrisoquine oxidation polymorphism has a strong impact on the metabolism of several drugs in patients, notably the TCAs (Bertilsson *et al.*, 1980, Brosen *et al.*, 1986a) and certain neuroleptics and cardiovascular drugs (Brosen and Gram, 1989a). A common property of substrates that are polymorphically metabolized by P450IID6 isozyme is that they have a basic nitrogen and are oxidized at a site with a distance of 0.5-0.7 nm from the basic nitrogen (Meyer *et al.*, 1986).

The frequency of the PM phenotype for debrisoquine (DQN) is approximately 8% in Western Europe and North America (Woolhouse *et al.*, 1979; Vinks *et al.*, 1982). In general, however, considerable interethnic variability exists in the debrisoquine PM frequency, ranging from 30% in a small Hong Kong population living in Canada (Kalow, 1982), 6-9% in Nigerians and Ghanians (Kalow, 1982; Idle and Smith, 1979), 1% Egyptians and Saudi Arabians (Mahgoub *et al.*, 1979; Islam *et al.*, 1980), to complete absence in a Japanese population (Nakamura *et al.*, 1985). Therefore, the frequency of PM phenotype for DQN appears to be markedly lower in non-Caucasian populations.

The pharmacogenetic probe drugs sparteine, DQN, dextromethorphan and bufuralol, and a host of widely used clinically important drugs (Brosen, 1990; Brosen and Gram, 1989a; Jacqz, 1986; Kalow, 1986; Robbins and Wedlund, 1990), are known substrates of P450IID6 isozyme (Table 1-5). It appears that these drugs have a much higher affinity for P450IID6 than for other P450 isozymes.

Table 1-5. List of some clinically used drugs known to be metabolized by P450IID6 isozyme

Ajmaline, alprenolol, amiflamine, AMI, bufuralol, captopril, chlorpromazine, CMI, clonidine, codeine, DQN, DMI, dextromethorphan, domperidone, encainide, flecainide, flurazepam, guanoxan, haloperidol, haloperidol epoxide, 4-hydroxyamphetamine, IMI, indoramin, labetolol, lidocaine, lobeline, *p*-methoxyamphetamine, methoxyphenamine, metiamide, metoprolol, mexiletine, nephazadone, NT, oxazepam, oxprenolol, penicillamine, perhexiline, perphenazine, phenacetin, phenformin, pindolol, pipamperone, propafenone, propranolol, *n*-propylajmaline, QNN, sparteine, temazepam, thioridazine, timolol, tomoxetine, yohimbine.

Metabolism of the drugs listed in Table 1-5 is markedly reduced in PMs, and the total clearance typically is about five times lower in PMs than in EMs. The difference is less pronounced when the drug is only partly biotransformed by P450IID6 (Brosen and Gram, 1989a). Another functional characteristic of P450IID6 appears to be non-linear kinetics for some drugs in EMs but linear in PMs. With oral dosing this probably reflects saturation of P450IID6 during the first pass through the liver (Brosen and Gram, 1988).

Clinical studies have demonstrated that PMs represent a high risk group in the population with a propensity to develop adverse drug effects (Meyer *et al.*, 1990). In general, the sparteine/DQN oxidation polymorphism is of significance in patient management only for those drugs for which plasma concentration measurements are considered useful and for which the elimination of the drug and/or its active metabolite is mainly determined by P450IID6. At present it applies to TCAs, certain neuroleptics and antiarrhythmics (Brosen and Gram, 1989a). Phenotyping should be introduced into clinical routine under strictly controlled conditions to afford a better understanding of its potentials and limitations. It would be of great value to identify PM and EM patients before treatment is started (Brosen, 1990).

The biotransformation of some drugs is not impaired in PMs of DQN. These drugs (Table 1-6) are oxidized by isozymes of P450 other than P450IID6 isozyme (Brosen and Gram, 1989a; Jacqz *et al.*, 1986; Kalow, 1986). Therefore, the sparteine/DQN oxidation polymorphism should not be expected to be of any significance in their clinical use (Brosen and Gram, 1989a). Codeine, however, is an exception to this statement; in EMs a maximum of 10% of codeine is O-demethylated to morphine, which is believed to be the active analgesic component. O-Demethylation is catalyzed by P450IID6, and this explains why codeine has no analgesic effect in PMs (Dayer *et al.*, 1988).

Table 1-6. List of some clinically used drugs known not to be metabolized by P450IID6 isozyme:

Acetanilide, alprenolol, amobarbital, antipyrine, atenolol, caffeine, carbamazepine, carbocysteine, carboxymethylcysteine, cholesterol, clonazepam, clozapine, codeine, cyclosporin, desalkylflurazepam, N-desmethyldiazepam, diazepam, erythromycin, ethinyloestradiol, ethosuximide, guanethidine, haloperidol, hexamethonium, hexobarbital, isoproterenol, lidocaine, maprotiline, mephenytoin, methaqualone, methylphenobarbital, metiamide, midazolam, nadolol, nicotine, nifedipine, ouabain, phenacetin, phenobarbital, phenylbutazone, phenylephrine, phenytoin, pinacidil, prazosin, primidone, procainamide, propranolol, QND, scopolamine, sotalol, sulphamethazine, theophylline, tolbutamide, triazolam, trimethadione, d-tubocurarine, valproic acid, verapamil.

1.5.2 Mephenytoin

Another drug oxidation polymorphism that has been revealed in studies of the 4-hydroxylation of S-mephenytoin (Kupfer and Preisig, 1984) is related to the activity of a different isozyme of P450, formerly known as P450meph (Meyer *et al.*, 1986), but now called P450IIC9 (Nebert *et al.*, 1989). About 5% of Caucasians and as many as 24% of Japanese are PMs of mephenytoin (Kupfer and Preisig, 1984; Jacqz *et al.*, 1985). Mephenytoin is virtually identical to mephobarbital which has an extra C=O group and now it is known that mephobarbital is also a substrate for P450IIC9 isozyme (Kupfer and Branch, 1985). The MAO-inhibiting antidepressant tranylcypromine (TCP) is able to bind quite strongly to P450IIC9 isozyme (Inaba *et al.*, 1985), so TCP might also be a substrate for this isozyme or an inhibitor of it (Kalow, 1986).

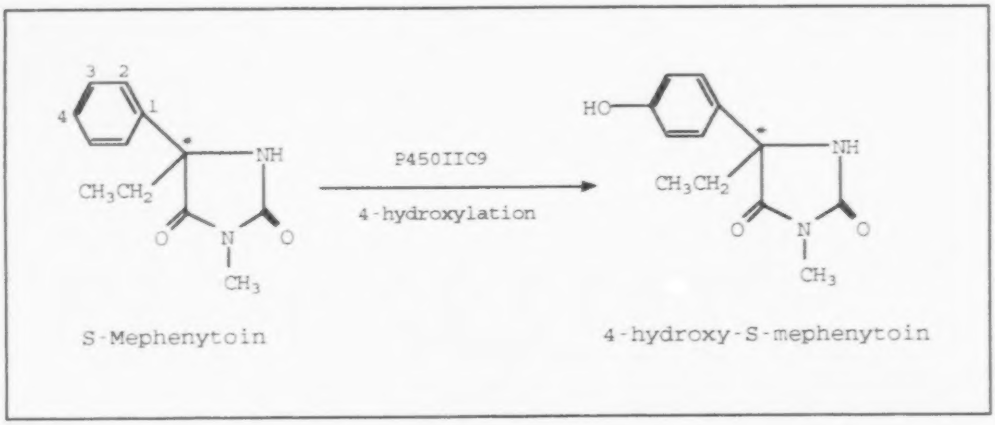

3
2
4
1
NH
CH3CH2
O
N
O
CH3
S-Mephenytoin
P450IIC9
4-hydroxylation
HO
NH
CH3CH2
O
N
O
CH3
4-hydroxy-S-mephenytoin

Figure 1-11. Metabolism of S-mephenytoin in man.

* Denotes asymmetric center

Interestingly, phenytoin, a structural analog of mephenytoin, is not a substrate for this isozyme (Inaba *et al.*, 1985).

Mephenytoin, mephobarbital and TCP all have a phenyl group attached to a ring system and the attachment is through an asymmetric center. It is not yet known whether this similarity in structure has anything to do with the enzyme system responsible for their metabolism (Coutts, 1991b). The broader clinical significance of the mephenytoin polymorphism has yet to be established.

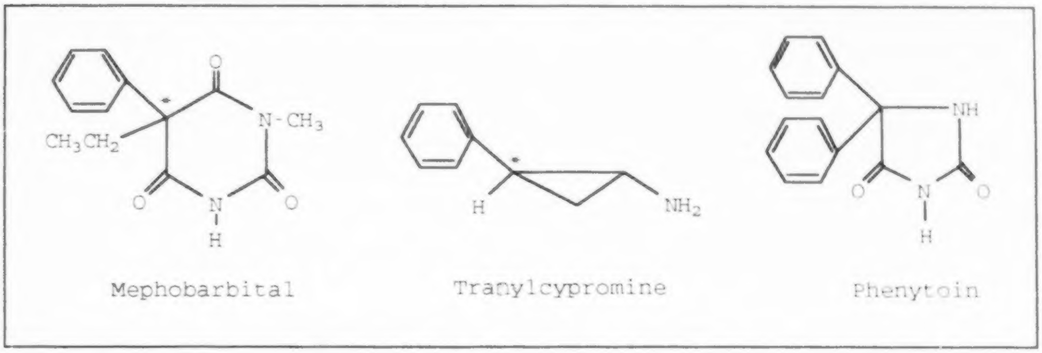


Figure 1-12. Chemical structures of mephobarbital, tranylcypromine and phenytoin.

* Denotes asymmetric center

A list of drugs metabolized by P450IIC9 is given in Table 1-7 (Inaba *et al.*, 1985; Jacqz *et al.*, 1986, Brosen, 1990).

Table 1-7. List of some drugs known to be metabolized by P450IIC9 isozyme.

Alprenolol, cortisol, cortisone, diazepam, N-desmethyldiazepam, estradiol, ethtoin, ethynylestradiol, flurazepam, hexobarbital, labetolol, mephenytoin, methsuximide, methylphenobarbital, nialamide, pimozide, propranolol, temazepam, testosterone, warfarin.

The results from the studies with P450 isozymes may in future allow prediction of the clinical pharmacokinetics of drugs, if their oxidation can be assigned to the activity of one or more well characterized P450s. This strategy will reduce animal experimentation and will also result in a safer and rational drug therapy (Brosen, 1990).

1.5.3 Phenotyping

Phenotyping is generally easy to perform but requires drug administration to humans and urine collection. Phenotyping during treatment with a potent inhibitor is uninformative. Sparteine, DQN or dextromethorphan may be used for the phenotyping procedure (Brosen and Gram, 1990). These drugs are oxidized rapidly and very specifically by P450IID6 isozyme (Brosen, 1990). In Canada and U.S.A., dextromethorphan is usually used for this purpose because of the general non-availabilty of sparteine and DQN.

The function of P450IID6 *in vivo* is simply expressed according to a metabolic ratio (MR):

MR=% of dose excreted as parent drug/% of dose excreted as metabolite(s).

1.5.3.1 Debrisoquine (DQN):

To phenotype an individual with this drug the following procedure is followed (Evans *et al.*, 1980; Idle and Smith, 1979). Here the urinary recoveries of DQN and its metabolite 4-OH-DQN are measured in 8-12h urine sample.

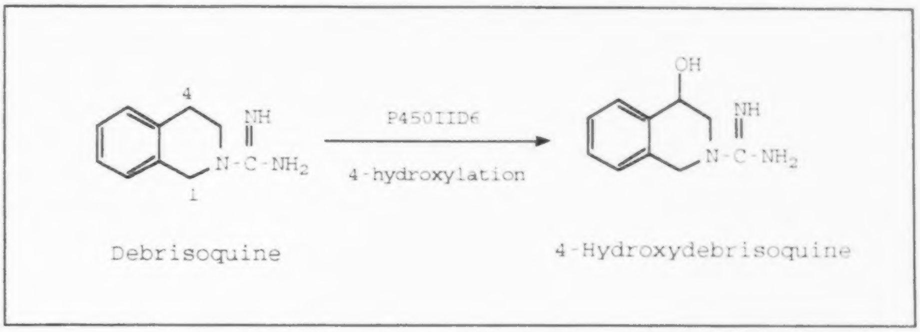

4
1
NH
N-C-NH2
P450IID6
4-hydroxylation
OH
NH
N-C-NH2
Debrisoquine
4-Hydroxydebrisoquine

Figure 1-13. Chemical structures of debrisoquine (DQN) and its major metabolite, 4-hydroxydebrisoquine

After emptying the bladder the person swallows one 10 mg Declinax (Roche) tablet. An 8-12h urine sample is then collected (Guttendorf *et al.*, 1990; Brosen, 1990; Brosen and Gram, 1989a).

For debrisoquine:

MR = % of dose excreted as unchanged DQN/% of dose excreted as 4-OH-DQN

A PM subject is defined as one whose MR in an 8-12h urine is greater than 12 ($\log_{10}$ MR > 1.10)(Evans *et al.*, 1980).

1.5.3.2 Sparteine (SPT)

In this case the urinary recoveries of SPT and its metabolites 2-dehydro-SPT and 5-dehydro-SPT are measured in

12h urine after an oral dose of 20mg SPT. If MR is greater than 20 the individual is a PM (Eichelbaum, 1982; Jacqz *et al.*, 1986).

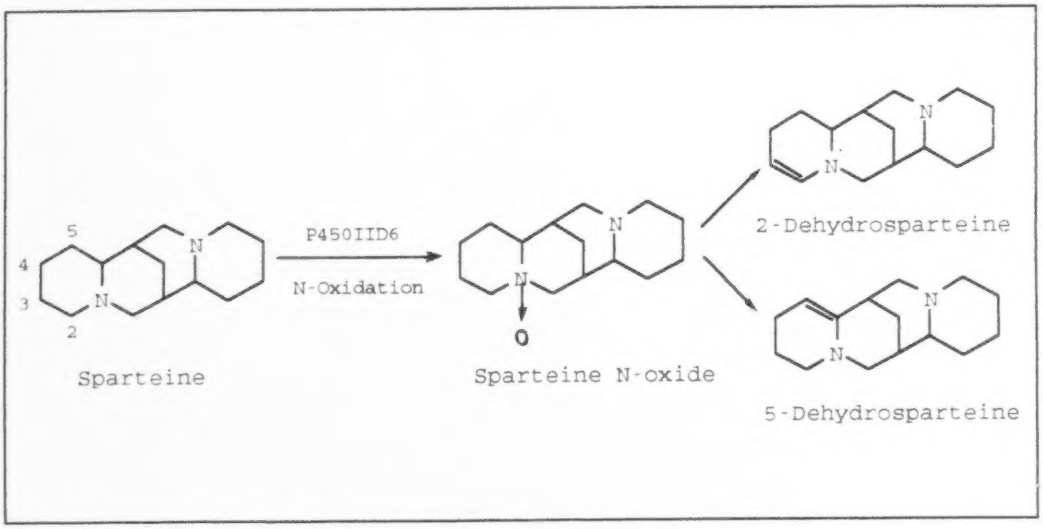

5
4
3
2
N
N
Sparteine
P450IID6
N-Oxidation
N
N
O
Sparteine N-oxide
N
N
2-Dehydrosparteine
N
N
5-Dehydrosparteine

Figure 1-14. Metabolic pathway of sparteine in man

1.5.3.3 Dextromethorphan (DM)

DM is now being used in Canada to phenotype individuals for hepatic cytochrome P450IID6 activity. Its major metabolic pathway is O-demethylation to dextrorphan (DR). This metabolic pathway of DM is catalyzed by the P450IID6 isozyme and is subject to polymorphic metabolism (Schimd *et al.*, 1985; Woodworth *et al.*, 1987; Vetticaden *et al.*, 1989).

The phenotyping procedure is similar to that of DQN. After emptying the bladder a single dose, equivalent to 60mg of DM, either as syrup or as tablet is administered to the person and a 8-12h urine sample is collected (Guttendorf *et al.*, 1990; Robbins and Wedlund, 1990). The urinary recoveries of DM and its metabolite dextrorphan (DR) arethen measured by HPLC or GC (Kupfer *et al.*, 1984; Henthron *et al.*, 1989; Robbins and Wedlund, 1990). The metabolic ratio (DM/DR) ranges from 0.0030 to 5.7 (Henthron *et al.*, 1989). A PM subject in this case is one whose MR is more than 0.30 (Schmid *et al.*, 1985; Guttendorf *et al.* 1990) or 0.26 (Henthron *et al.*, 1989).

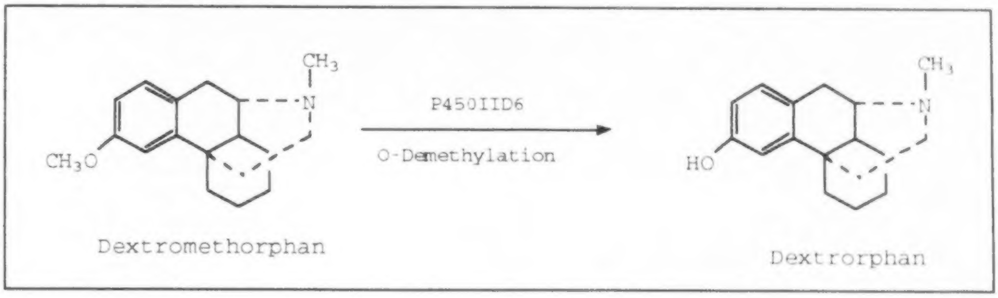


Figure 1-15. Structures of dextromethorphan (DM) and its major metabolite dextrorphan (DR)

Any of the following single-ingredient cough syrups could be used for phenotyping:

Anti-Cough syrup® (Lee-Adams), Balminil DM syrup® (Rougier), Benylin DM® (Parke-Davis), Koffex® (Rougier), Robidex® (Robins), Sedatuss® (Trianon), Vicks Children's Cough Syrup®, or another commercially available DM (single active ingredient) product.

1.6 Analytical Procedures

Analytical methods for several compounds will be described in this thesis. GC techniques have been developed for the simultaneous analysis of drugs and their metabolites in biological fluids. A GC-MS technique was used for identification and structural characterization of drug metabolites.

1.6.1 Isolation and purification of drugs and their metabolites from biological fluids

The study of xenobiotic metabolism has developed over the last few years into a specialized science with its own technical skills, three of which are the isolation, identification and quantitation of metabolites. Drugs can be classified as acidic, basic, amphoteric or neutral on the basis of their pKa values. The TCAs are mostly lipophilic bases with pKa values of 8.5-10 or higher. Most extraction procedures take advantage of these properties to separate the drug and its metabolites from the biological matrix. A three step liquid-liquid extraction procedure is the most commonly used method for the initial purification (Reed, 1988). This involves first adjusting the pH of the sample to >10 with sodium hydroxide solution and extracting with a suitable organic solvent. The organic layer, which now contains the analytes, is dried under vacuum or under a stream of dry nitrogen. The residue obtained is dissolved in dilute hydrochloric acid and then washed with a suitable organic solvent. The acidic aqueous phase which now contains the basic drug and basic metabolites is made alkaline by the addition of ammonium hydroxide and the analytes are extracted into a suitable organic solvent. The organic layer is evaporated as described above and finally the residue obtained is derivatized by a suitable derivatizing agent before being analyzed by GC or other analytical procedure.

Solid phase extraction techniques have recently been proposed for extracting antidepressants from their biological matrix (Narasimhachari *et al.*, 1981; Skrinska *et al.*, 1984). The technique involves the use of a small column containing a packing material such as C_{18} bonded silica. These small columns are first washed with methanol, then an acidic buffer containing approximately 5% methanol, followed by sample. Analytes are then collected by elution with a small amount of organic solvent such as methanol. The sample can now be analyzed after appropriate chemical derivatization.

1.6.2 Derivatization technique

The scope of biochemical GC would be quite limited without sample derivatization. For example, benzoic acid because of its polarity is not suitable for gas chromatographic analysis, but derivatization with methanol in presence of boron trifluoride readily converts it to methyl benzoate, which is more stable and volatile and easier to chromatograph. Therefore, analytes are derivatized for several reasons (Coutts and Baker, 1982; Reidman M, 1973):

a) to increase volatility

b) to increase stability

c) to reduce the polarity

d) to introduce specific groups which enhance detectability

e) to increase extractibility from aqueous solutions

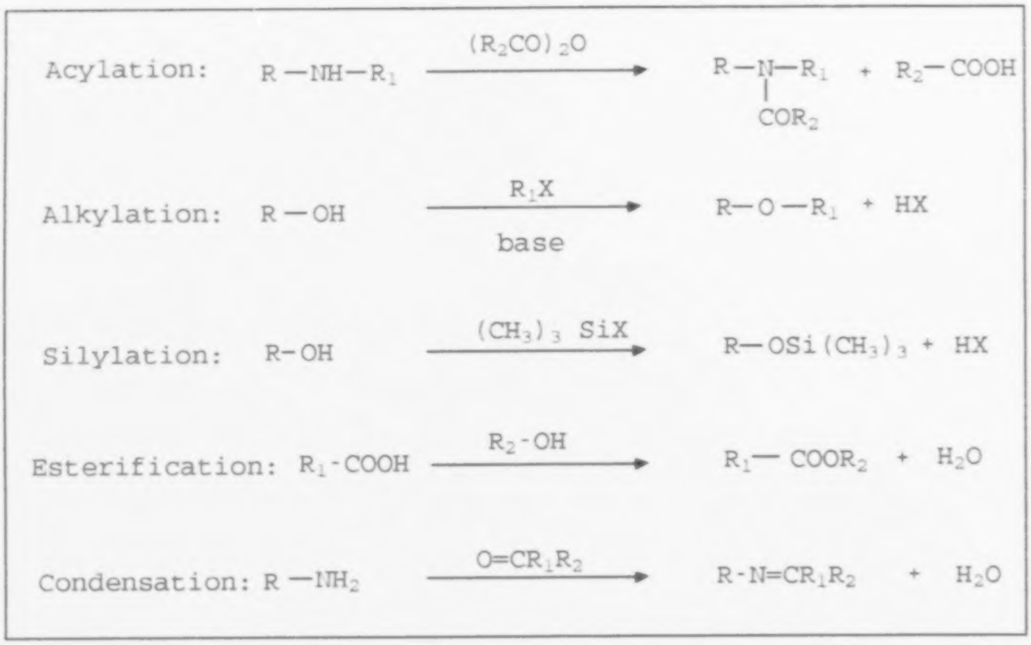
f) to improve chromatographic separation of different components present in the sample mixture

Derivatization of amines, alcohols and carboxylic acids reduces the adsorption of these compounds by the GC column (Vandenheuvel and Zacchei, 1976). Most derivatization reactions involve the replacement of active hydrogen in polar groups (*e.g.* NH, OH, COOH), and the main types of derivatizations are alkylation, acylation, silylation and condensation (Knapp, 1979; Blau and King, 1978; Coutts and Baker, 1982). Figure 1-16 illustrates the principal types of derivatization used in GC analysis (Baker *et al.*, 1982).

1.6.3 Gas chromatography

Adsorption chromatography was first applied by Tswett in 1906, and in 1952 James and Martin introduced gas-liquid chromatography (Ettre and Horvath, 1975). Gas chromatography, also known as gas-liquid chromatography (GLC), is an analytical procedure used to separate mixtures of organic compounds prior to their identification and/or quantitation. The volatile components of an analyte mixture are carried through a column by an inert carrier gas (called mobile phase) and the components are separated from one another according to their partition coefficients between the carrier gas and the liquid stationary phase. Gas chromatography can be used for the analysis of gas, liquid, and solid samples, providing that the latter two can be thermally vaporized without significant decomposition (Kline and Soine, 1984).

The choice of the GC columns depends on the polarity of the compound under investigation. Much better resolution is attained on capillary columns than on packed columns, so capillary columns are now being used increasingly. The most common column used in the analysis of psychotropic drugs is OV-17 or DB-17 (Cooper, 1988).



Acylation: $$R-NH-R_1 \xrightarrow{(R_2CO)_2O} R-\underset{\displaystyle COR_2}{\underset{|}{N}}-R_1 + R_2-COOH$$

Alkylation: $$R-OH \xrightarrow[\text{base}]{R_1X} R-O-R_1 + HX$$

Silylation: $$R\text{-}OH \xrightarrow{(CH_3)_3\ SiX} R-OSi(CH_3)_3 + HX$$

Esterification: $$R_1\text{-}COOH \xrightarrow{R_2\text{-}OH} R_1-COOR_2 + H_2O$$

Condensation: $$R-NH_2 \xrightarrow{O=CR_1R_2} R\text{-}N=CR_1R_2 + H_2O$$

Figure 1-16. Principal types of derivatization procedures used in GC analyses.

Compounds eluting from a gas chromatograph are detected by a variety of techniques. The choice of GC detectors depends on the properties of the analyte itself and of its derivatives. The flame ionization detector (FID) is an universal detector, while the electron capture detector (ECD) and nitrogen-phosphorus detector (NPD) are more selective and sensitive. The minimum detection limit for ECD and NPD is in the picogram range (Kline and Soine, 1984). The NPD has been extensively used for the analysis of TCAs (Cooper, 1988).

1.6.4 Gas chromatography-Mass spectrometry

A GC-MS analytical technique was first used by Hammer *et al.* in 1968 for the analysis of chlorpromazine and some of its metabolites isolated from human blood (Hammer *et al.*, 1968). Since that time this methodology has been used successfully for the analysis of drugs and their metabolites in biological fluids. When MS is used in combination with the excellent separating power of GC, the combination provides an analytical technique that is highly selective and sensitive and has the versatility to permit quantitation and identification of the structures of drugs and their metabolites. These properties make GC-MS the method of choice in drug metabolism studies (Reed, 1988). Metabolic studies utilizing combined GC-MS have been reported for drugs from almost every pharmacological category (Cone, 1984). Indeed, GC-MS has become almost a routine technique for the identification and quantitation of a wide range of compounds of biological interest (Millard, 1976; Maurer and Pfleger, 1984)

Complex mixtures of drugs, metabolites and endogenous compounds are first separated on a GC, and the effluent from the GC is fed through an interface to the ionization chamber of a MS. Ionization of the compounds then can be accomplished by one of several methods available, *e.g.* electron ionization (EI), chemical ionization (CI), fast atom bombardment (FAB) etc. The fragmented ions are separated according to their mass-to-charge ratio (*m/z*) and collected. The resultant signal is usually displayed in the form of a spectrum, called a mass spectrum. Detailed interpretation of the mass spectral fragmentation pattern reveals the structural identity of the drug and its metabolites.

1.7 *In vitro* and *in vivo* animal models of drug metabolism

1.7.1 *In vitro* models

Drug metabolism can be studied either *in vivo*, *i.e.*, whole body experiments, or *in vitro* where specific enzyme systems are isolated from tissues or organs. *In vitro* metabolism studies reveal the liver's or other organ's contribution to the metabolism of a drug substrate.

The major purposes of *in vitro* drug metabolism studies are:

1) to determine in which tissues drug metabolism takes place

2) to determine the steps involved in the formation of metabolites

3) to discover the components of the enzyme systems that catalyze these various steps

To achieve these objectives, investigators may choose from a variety of tissue preparations and techniques, each of which has its advantages and disadvantages (Gillette, 1971).

1.7.1.1 Preparation of liver microsomes

Azo-reduction and N-demethylation of aminoazo dyes were among the first examples of *in vitro* metabolism of xenobiotics by liver homogenates (Mueller, 1949 and 1953). These experiments and others demonstrated that there was a specific fraction of liver homogenate that could be obtained by differential centrifugation and was responsible for the metabolism observed. This specific portion of the homogenized liver is now referred to as liver microsomes or microsomal fraction or the 10,000 g supernatant and is routinely used in *in vitro* drug metabolism studies (Mazel, 1971). A typical procedure is now described.

After an overnight fast, the rat (or other animal) is sacrificed then exsanguinated. Fasting reduces the glycogen content of the liver and thereby prevents losses of microsomes during centrifugal separation procedure (Dallner *et al.*, 1966). The liver is then removed, washed with isotonic (1.15%) KCl solution, blotted dry and weighed. The following operations are carried out in the cold (4-6°C). Portions (5g) of minced liver are homogenized for 1 min with a glass Potter-Elvehjem homogenizer in 3 volumes (25% w/v) of ice-cold isotonic (1.15%) KCl-0.01M phosphate buffer, pH 7.4 (Bickel and Baggiolini, 1966; von Bahr *et al.*, 1980; Coutts *et al.*, 1981; Satterwhite and Boudinot, 1991). The crude homogenate is then centrifuged at 10,000 g for 20 min at 5°C to remove nuclei and mitochondria (Mazel, 1971). The 10,000 g supernatant fraction contains microsomes and soluble proteins. A 1.0 ml portion of this microsomal preparation is equivalent to 250 mg of liver. A schematic representation of the procedure used to prepare various fractions of liver is shown in Figure 1-17. Although *in vitro* metabolism studies generally employ liver preparations, microsomal fractions can also be obtained from other tissues *e.g.* lung, adrenals, brain, testes and kidney (Orrenius *et al.*, 1973; Bend *et al.*, 1973).

The microsomal fraction is not the only portion of the liver homogenate that is of interest in drug metabolism. Purified microsomal protein is obtained by ultracentrifugation (100,000 g for 60 min) of the 10,000 g supernatant (Fig. 1-17). The so-called microsomal pellet obtained by this method is used to study the properties of cytochrome P450 (Omura and Sato, 1964) and to determine microsomal protein (Lowry *et al.*, 1951; Miller, 1959).

It was demonstrated by Brodie *et al.* (1955) that the reduced form of nicotinamide adenine dinucleotide phosphate (NADP), *i.e.* NADPH, was essential for the activity of the microsomal system. NADPH is generated *in situ* from NADP and glucose-6-phosphate (G-6-P) in the presence of enzyme G-6-P dehydrogenase (Gibson and Skett, 1986). G-6-P dehydrogenase is present in the 10,000 g microsomal preparation but is lost when 100,000 g pellet is prepared. Furthermore, the 10,000 g fraction is much easier to prepare, the reaction is linear for a long period of time and the enzymic activity obtained is usually greater than that which can be achieved with 100,000 g microsomal pellets plus a NADPH-generating system. Therefore, for many metabolism studies there are advantages in using the 10,000 g supernatant rather than isolated microsomes (*i.e.* 100,000 g pellets)(Mazel, 1971).

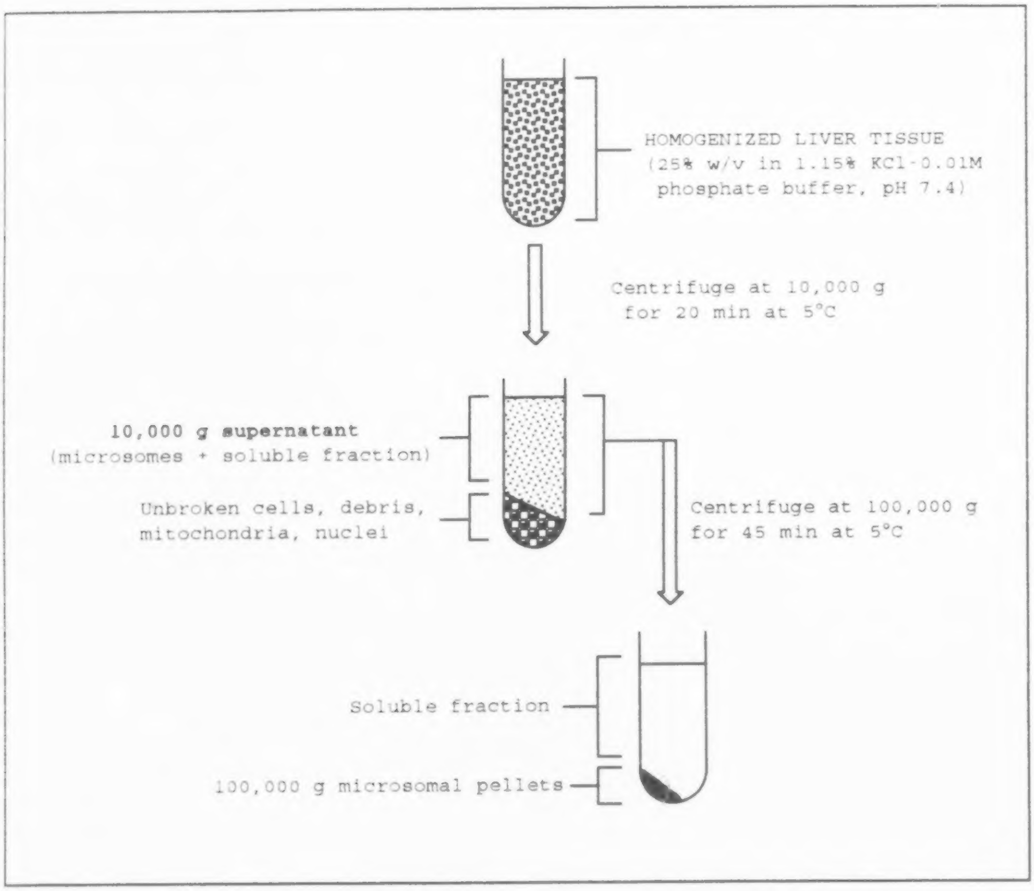

HOMOGENIZED LIVER TISSUE
(25% w/v in 1.15% KCl-0.01M phosphate buffer, pH 7.4)
Centrifuge at 10,000 g for 20 min at 5°C
10,000 g supernatant
(microsomes + soluble fraction)
Unbroken cells, debris, mitochondria, nuclei
Centrifuge at 100,000 g for 45 min at 5°C
Soluble fraction
100,000 g microsomal pellets

Figure 1-17. Schematic representation of the procedure used to prepare various fractions of liver homogenate for use in *in vitro* drug metabolism studies.

1.7.2 *In vivo* animal models

1.7.2.1 Intact animals

While *in vitro* studies are required to understand individual organs' contribution to metabolism of any xenobiotic, ultimately *in vivo* (studies on intact live animals) metabolism studies are necessary to establish different biotransformation pathways and to determine various factors which affect the levels of drugs and their metabolites. One of the problems of *in vivo* studies is to identify a suitable animal model. The difficulties of extrapolation of animal data to human has stimulated the search for suitable animal models.

Male Sprague-Dawley (MSD) rats were chosen in this thesis research for the following reasons:

1. Cytochrome P450IID6 isozyme, which catalyzes the metabolic oxidation of most basic drugs, is present in MSD rats (Gonzalez *et al.*, 1987).
2. The rat is the animal most commonly used for metabolic studies.
3. The rat provides a steady flow of bile throughout the 4-8h duration of an experiment.
4. Rats are easy to handle and to house.

1.7.2.2 Bile duct cannulated animals

The route of excretion of a xenobiotic is related to its lipophilicity. Low molecular weight hydrophilic metabolites are excreted mainly in urine. The threshold for biliary excretion is dependent upon animal species and polarity and molecular weight of a compound. The threshold molecular weight for rat is 325 ± 50, for guinea pig 400 ± 50, and for rabbit 475 ± 50 (Martin and Reid, 1981). Compounds of intermediate polarity and molecular weight may be excreted in both urine and bile. The rat is a particularly useful animal for biliary excretion studies, but it is known that rat generally excretes substances more readily in bile than does man (Klaassen *et al.*, 1981). The rat does not possess a gall bladder and it is possible to cannulate the bile duct and exteriorize the cannula. In this thesis research hepatobiliary excretion of three drugs, namely TMP, IPR and IMI, was investigated.

Since the microflora present in the gastrointestinal tract can metabolize xenobiotics, the presence of metabolites in feces does not indicate they were present in the body *per se*..For this reason the excretion of drugs and metabolites into bile and not feces was investigated.

After a compound is secreted into bile and enters the small intestine, it is either eliminated in the feces or reabsorbed from the intestine. Compounds which are secreted into bile and reabsorbed from the intestine are said to undergo enterohepatic circulation. Lipid-soluble compounds are more readily absorbed from the intestine than the hydrophilic metabolites and thus undergo a more extensive enterohepatic circulation than water-soluble compounds. Although hepatic biotransformation to a more polar form decreases the enterohepatic circulation, intestinal bacteria are sometimes able to convert the water-soluble metabolites back to their lipid-soluble form, which enhances intestinal reabsorption (Williams *et al.*, 1965). Thus, alterations in intestinal flora with antibiotics may decrease the enterohepatic circulation of some xenobiotics and concurrently shorten the pharmacological half-life of the drug (Williams *et al.*, 1965). Since, IMI, an analog of TMP, is excreted in the bile and undergoes enterohepatic circulation (Dencker *et al.*, 1976), it is possible that TMP also follows the similar processes.

2. RATIONALE, HYPOTHESES, AIMS AND OBJECTIVES

2.1 Rationale for the thesis research

TMP has been used as an antidepressant for many years. IPR has also been used clinically for this purpose, but to a lesser extent than TMP; however, it has been employed extensively as a pharmacological tool to block ring hydroxylation. Despite this, there is a paucity of information on the metabolic pathways of both drugs.

2.1.1 Trimipramine

To date no comprehensive metabolic study has been reported for TMP in the rat and its metabolism in man has been reported only recently (Cowan *et al.*, 1988; Maurer, 1989). In addition to being effective in the treatment of clinical depression, TMP is believed by some to be a potent histamine H_2-receptor antagonist and also to be effective in the treatment of duodenal and gastric ulcer (MacKay *et al.*, 1984). TMP is also claimed to reduce joint pain and tenderness significantly in selected rheumatoid arthritic patients (MacFarlane *et al.*, 1986). These diverse pharmacological properties of TMP may be associated with some of its many metabolites. To date no research regarding the alicyclic hydroxylation of TMP in man has been reported. Hydroxylated and N-demethylated metabolites of ADs are pharmacologically active and it was of interest to see whether these species are formed in man and in rat.

2.1.2 Drug-drug interaction studies

Metabolic oxidations of most basic drugs are catalyzed by the cytochrome P450IID6 isozyme system. Aromatic hydroxylation of the structurally related drugs, IMI and DMI for example, is catalyzed by P450IID6. It was important to know whether this polymorphic enzyme system was also involved in the aromatic hydroxylation of TMP. The antiarrhythmic drug QND is a known competitive inhibitor of P450IID6. It was proposed to study the effects of QND and other known inhibitors on the metabolism of TMP, so as to determine P450IID6 isozyme's contribution to the metabolism of TMP.

2.1.3 Iprindole

The metabolism of IPR is only poorly understood. Though the drug has been marketed for more than 20 years, surprisingly only one metabolic study has so far been reported. What is known about IPR is its ability to inhibit the aromatic hydroxylation of amphetamine, leading to increased tissue levels of amphetamine (Freeman and Sulser, 1972). Recently it has been reported that IPR is also a potent inhibitor of N-dealkylation of fenfluramine (Hegadoren *et al.*, 1990). Many other drugs, including IMI, DMI, TMP, are metabolized to ring hydroxylated and N-dealkylated products, and the prior or concurrent administration of IPR with any of these ADs could be expected to alter the relative importance of the antidepressants' metabolic pathway.

Prior to commencing such drug/drug interaction studies involving TMP and IPR in the rat, however, it was essential to be informed of the metabolic pathways of IPR in that species.

2.1.4 Biliary excretion studies

Knowledge of the biliary route of excretion of drugs lags behind that of renal excretion. The relative inaccessibility of bile is probably the major reason for this slow progress. Little is known about the biliary excretion of TMP and IPR in mammals. Knowledge of the biliary excretion of a drug is important for a variety of reasons:

1. Biliary metabolites can undergo enterohepatic recirculation

2. Enterohepatic recirculation could increase the half-life of a drug and/or metabolites and may lead to possible toxicity

3. Biliary excretion becomes important when the hepatic clearance of a drug is appreciable

4. To calculate the mass balance of a drug and its metabolites if that drug is excreted in bile as well as by the kidney

For these reasons, studies were also designed to identify the biliary metabolites of TMP, IMI and IPR in the rat.

2.2 Hypotheses

1. TMP undergoes metabolic aromatic hydroxylation and N-dealkylation in man. Since the MSD rat possesses P450IID2 and P450IID6 isozymes, similar metabolic profiles are expected for TMP in the MSD rat.

2. Structurally similar drugs, IMI, DMI and AMI, undergo alicyclic hydroxylation in man. Therefore, it is proposed that TMP undergoes alicylic hydroxylation in man.

3. Cytochrome P450IID6 isozyme is involved in the aromatic hydroxylation of IMI and DMI. Therefore, aromatic hydroxylation of TMP is expected to be catalyzed by P450IID6.

4. QND and QNN, competitive inhibitors of cytochrome P450IID6 isozyme, significantly inhibit the aromatic hydroxylation of IMI and DMI. Therefore, decreased aromatic hydroxylation of TMP is expected when rats are pretreated with either QND or QNN.

5. Since IPR acts as a competitive inhibitor of aromatic hydroxylation of AMP and other drugs, its prior administration should decrease the aromatic

hydroxylation of TMP. IPR might be a competitive inhibitor of P450IID6 isozyme.

6. Some tertiary amine TCAs undergo biliary excretion. It is proposed that TMP and some of its lipophilic metabolites also undergo biliary excretion. The extent of biliary excretion may differ from that of other ADs.

7. IPR undergoes alicyclic oxidation, N-demethylation and aromatic oxidation in monkeys and pigs. Some of these metabolic pathways are expected for IPR in the MSD rat.

8. Derivatization techniques are suitable methods with which to identify the sites of metabolic oxidation in drugs.

2.3 Aims and objectives

The primary objectives of the thesis research are to isolate, identify, characterize and quantify both *in vitro* and *in vivo* metabolites of TMP and IPR , two widely used and yet little investigated TCAs, using suitable extraction, derivatization and computerized GC-MS method and other analytical techniques. The pathways of formation of some of these metabolites will also be studied. The metabolism of N-n-butylamphetamine (NBA) and IMI are also investigated for comparison purposes.

Cytochrome P450 isozymes play a vital role in the metabolism of many basic drugs. It is of interest to establish their role in the metabolism of TMP. Concomitant administration of drugs sometimes results in adverse side effects. It is pertinent to study the effects of the known inhibitors of P450 isozymes on the metabolism of TMP and other substrates.

During the course of this research work several assay methods had to be developed for the simultaneous determination of drugs and their metabolites in biological matrices. To enhance the identification of the sites of metabolism, suitable derivatization methods were also developed.

Phenotyping is a vital tool for rational drug therapy of some drugs whose metabolic oxidation is polymorphic. It was necessary to develop an easy and rapid method of phenotyping individuals for P450IID6 isozyme activity.

The aims and objectives of the present thesis research may be summarized:

1. Development of suitable extraction and purification procedures for TMP, IPR, IMI and NBA and their metabolites from biological matrices.
2. Development of suitable derivatization procedures for drug metabolites for GC and GC-MS analyses.
3. Development of specific and sensitive GC assay methods for both qualitative and quantitative determination of drugs (TMP, IPR, IMI and NBA) and their metabolites.

4. Development of suitable GC-MS methods for the structural characterization of the above drugs and their metabolites.
5. Investigation of both *in vitro* and *in vivo* metabolism of TMP in the rat.
6. Investigation of *in vivo* metabolism of TMP in man.
7. Delineation of pathways of formation of some *in vivo* metabolites of TMP in the rat.
8. Studies on *in vitro* and *in vivo* biotransformation of IPR in the rat.
9. Delineation of the effects of QND, QNN and IPR on the metabolism of TMP in the rat.
10. Determination of the effects of QND on the metabolism of NBA.
11. Investigation of the role of the cytochrome P450IID6 isozyme on the aromatic oxidation of TMP and NBA.
12. Characterization of the biliary metabolites of TMP and IPR in the rat.
13. Quantitation of the *in vivo* metabolites of IMI in human.
14. Development of a simple and rapid GC analytical technique to phenotype individuals for hepatic cytochrome P450IID6 activity.

3. MATERIALS AND METHODS

3.1 Chemicals and biologicals

3.1.1 Commercial sources

The following chemicals and biologicals were obtained from commercial sources.

Materials	*Suppliers*
Dichloromethane, diethyl ether, ethyl acetate, hydrochloric acid, methanol, pyridine, sodium hydroxide	BDH Chemicals Canada Limited, Toronto, Ontario, Canada
Acetic anhydride, isopropanol, magnesium chloride, normal saline, potassium dihydrogen phosphate, sodium acetate, sodium bicarbonate, sodium phosphate dibasic, trichloroacetic acid,	Fisher Scientific Co., Fair Lawn, NJ, USA
Aryl sulfatase type VIII, glucose-6-phosphate, glucose-6-phosphate-dehydrogenase β-glucuronidase type VII, β-glucuronidase type H-1	Sigma Chemical Co., St.Louis, MO, USA
Nicotinamide adenine dinucleotide phosphate-sodium salt	Terochem Laboratories, Edmonton, Alberta, Canada

Nicotinamide	Mann Research Laboratories Inc., New York, NY, USA
Protein Assay Kit (#5656)	Sigma Diagnostics, St.Louis,MO, USA
Air, Helium, nitrogen, and hydrogen gas	Linde®, Union Carbide Canada Ltd., Toronto, Ontario, Canada
Disposable syringes (1 ml, 5 ml and 10 ml), Needles (18 G, 23 G and 26 G), PE-10 and PE-50 tubing	Becton Dickinson and Company, Rutherford, NJ, USA

3.1.2 Gifts

Trimipramine (TMP) maleate and its metabolites norTMP maleate, 2-OH-TMP fumarate and 2-OH-norTMP fumarate were gifts from Rhône-Poulenc Pharma, Inc., Montréal, Canada. Iprindole hydrochloride was generously supplied by Wyeth Research UK Ltd., Berkshire, England. Fluoxetine hydrochloride was a gift from Eli Lilly Canada Inc., Scarborough, Ontario, Canada. N-*n*-Butylamphetamine (NBA), 4-OH-NBA, 4-OH-3-OCH$_3$-NBA and 4-OCH$_3$-NBA were provided by Dr. R.T. Coutts. Imipramine (IMI) hydrochloride and its metabolites desipramine (DMI) hydrochloride, 2-OH-DMI oxalate, 2-OH-IMI hydrochloride and 10-OH-IMI hydrochloride were supplied by Ciba-Geigy Canada, Mississauga, Ontario, Canada.

3.1.3 Distilled water

Water was distilled and deionized by a Milli-Q® Reagent Water System, purchased from Millipore Corporation, Bedford, Mass. USA.

3.1.4 Solvent distillation

All solvents (diethyl ether, ethyl acetate, dichloroethane, isopropanol) were glass distilled and stored in glass containers with ground glass stoppers.

3.2 Instrumentation

3.2.1 Gas chromatography

3.2.1.1 Nitrogen phosphorus detection

Gas chromatographic analyses with nitrogen-phosphorus detection (NPD) was performed on a Hewlett Packard (HP) 5730A gas chromatograph (Palo Alto, CA, USA) equipped with HP 18740B capillary column control and a HP 7671A automatic sampler. The GC was interfaced with a HP 3396A electronic integrator for peak height or peak area measurements.

3.2.1.2 Flame ionization detection

For flame ionization detection (FID) a HP 5700 gas chromatograph was used. The chromatograph was equipped with a HP 18740 capillary column control and connected to a HP 3390A electronic integrator.

3.2.1.3 Electron capture detection

A Perkin Elmer model Sigma 3B gas chromatograph equipped with a 15 mCi ^{63}Ni source was employed for electron capture detection. The chromatograph was interfaced with a HP 1940A auto sampler and a HP 3390A integrator. Helium at a flow rate of 1 ml/min was used as a carrier gas and 5% methane in argon at a flow rate of 35 ml/min was employed as a makeup gas.

3.2.1.4 Mass spectrometer detection

A Varian Vista 6000 GC (Varian Instrument, Sunnyvale, CA, USA) was employed when a VG 7070E mass spectrometer was used as a detector.

Carrier gas

Helium at a flow rate of 1ml/min was employed as a carrier gas in all the cases. The make up gas was also helium, except for ECD, and the flow rate was 35ml/min. The injection port and detector temperatures were maintained at 250°C and 300°C respectively, but the oven temperature programming conditions were changed depending upon the analytical requirements and are described in the individual assay methods.

3.2.1.5 GC columns

Depending on the analytical requirements, different fused silica capillary columns were employed. They were:

1. DB-17 capillary column having 100% methylphenyl polysiloxane crosslinked and bonded stationary phase, i.d. 0.31 mm, film thickness 0.5µm, length 30m.
2. DB-17 capillary column having 100% methylphenyl polysiloxane crosslinked and bonded stationary phase, i.d. 0.20 mm, film thickness 0.25µm, length 20m.
3. DB-5 capillary column with 95% dimethyl-5% diphenyl polysiloxane crosslinked and bonded stationary phase, i.d. 0.20 mm, film thickness 0.25µm, length 30m.
4. DB-1 capillary column having 100% dimethyl polysiloxane crosslinked and bonded stationary phase, i.d. 0.20 mm, film thickness 0.25µm, length 30m.

3.2.2 Gas chromatography-Mass spectrometry

Gas chromatography-mass spectrometry was employed to determine the structures of drugs and their metabolites. These GC-MS analyses were performed using the same column that had been employed for GC analyses under identical temperature conditions. Electron ionization mass spectra (EIMS) were recorded in two types of GC-MS systems:

1. A quadropole HP 5980A mass spectrometer interfaced with a HP 5710 gas chromatograph and an 5934A 21 MX computer for data processing.

2. A magnetic deflection VG-7070E mass spectrometer (VG, Manchester, UK) linked to a Varian Vista 6000 gas chromatograph and a PDP 11 data system (Analytech Instrumentation and Service, St. Laurent, Canada).

3.2.3 Tissue homogenizer

Homogenization of the liver samples was performed utilizing a glass Potter-Elvehjem homogenizer and Teflon® pestle (Eborbach Corporation, Ann Arbor, MI, USA). The homogenization was done in a cold room (4-6°C) and the homogenizer tube was immersed in ice during homogenization. Care was taken to avoid prolonged homogenization time, which leads to overheating and possible enzyme destruction (Mazel, 1971).

3.2.4 Centrifuges

Different types of centrifuges were employed depending on the need of the experiment. All low speed (up to 1500 g) centrifugations were performed on either a Dynac® centrifuge (Clay Adams, Becton, Dickinson and Co. Parsippany, NJ, USA) or Beckman Microfuge (Beckman, Spino Division, Palo Alto, CA, USA). A refrigerated IEC B-20A centrifuge (Damon/IEC Division, Needham Hts., Mass., USA) was used to prepare 10,000 g microsomal preparation. For making 100,000 g microsomal pellets a Beckman L5-50 refrigerated ultracentrifuge (Beckman Instruments Inc., Spinco Division, Palo Alto, CA, USA) was employed.

3.2.5 Shaker-Mixer

An electronic IKA-VIBRAX-VXR® (Jane and Kunkel GmbH, Sweden) tube shaker was used for extraction purposes. It has the capacity of holding 36 test tubes. For vortexing individual test tubes a Vortex-Genie® model K-550G (Scientific Industries Inc., Bohemia, NY, USA) was employed.

3.2.6 Other instruments

A Pierce Reacti-Therm® heating module (Pierce Chemical Company, Rockford, IL, USA) was used for heating tubes during derivatization procedures. For incubation purposes during *in vitro* metabolism studies, a Magni Whirl® Constant temperature bath (Blue M Electric Company, Blue Island, IL, USA) was employed. A Fisher Accumet® pH meter, model 140 (Fisher Scientific Company, Fair Lawn, NJ, USA) was utilized for pH measurements.

3.4 Glassware cleaning

All glassware was thoroughly cleaned and dried before use. The procedure involved washing the glassware several times with water and scrubbing with plastic bristle brush without using soap or detergent. The glassware was then rinsed with distilled water followed by methanol and finally with dichloromethane. The glassware was allowed to air dry and then heated at 300°C in a Temco® muffle furnace (Thermo Electric Mfg. Co., Dubuque, IA, USA) for at least 2h.

Hamilton® glass syringes (Hamilton Co., Reno, NV, USA) were employed for sample injection and were periodically cleaned with 3M HCl followed by successive rinses with distilled water, methanol and ethyl acetate.

3.5 Animals

Male Sprague-Dawley rats were used in all the experiments described in this thesis. Rats (175-450g) were purchased from the Health Sciences Laboratory Animal Services, B-109 Medical Sciences Building, University of Alberta, Edmonton, Alberta. Animals were housed in plastic cages on cedar chip bedding in a temperature controlled room. A 12h light/dark cycle was maintained throughout the duration of the experiment. Water and Lab Chow (Wayne Rodent Blox, Tekland/Premier Laboratory Diets, Bartonville, IL, USA) were provided *ad libitum*. During metabolism study, rats were housed individually in plastic metabolic cages (Nalgene®, model E 1000, Maryland Plastic Inc.,New York, NY, USA) designed for separate collection of urine and feces.

Procedures involving use of rats were approved by the University of Alberta Health Sciences Animal Welfare Committee and were conducted according to the guidelines established by the Canadian Council on Animal Care.

3.6 Liver samples

Rat liver samples were obtained from MSD rats as described in section 1.7.1. The liver was either used immediately or stored at -80°C until the time of the experiments.

3.7 *In vitro* studies

3.7.1 Preparation of rat liver microsomes

In vitro studies were performed to determine hepatic contribution to overall metabolism of a particular substrate, as well as to have a prior idea of the nature of the metabolites before *in vivo* studies were conducted. Rat liver microsomes were prepared by a method similar to that described in section 1.7.1.1.

Portions of the liver microsomes were centrifuged at 100,000 g for 45 min at 4°C to obtain microsomal pellets. The microsomal pellets were used for protein determination and the liver microsomes were utilized in drug metabolism studies. Protein content was determined by a modified Lowry method using Sigma Protein Assay Kit # P5656 (Sigma Chemical Co. St.Louis, MO, USA).

3.7.2 Incubation procedure

The standard incubation mixture contained 300 nmol substrate (TMP or IPR), 25 μmol magnesium chloride, 50 μmol nicotinamide, 2 μmol NADP, 10 μmol G6P in a final volume of 3 ml. The above mixture was preincubated (Gibson and Skett, 1986) in an open 25 ml Erlenmyer flask at 37°C for 2 min in a shaking Magni Whirl® constant temperature bath at 120 oscillations/ min. The enzyme reaction was started by adding 1 ml of liver microsomes and incubation was continued for different time intervals, depending on the type of experiment. The reaction was stopped by adding 500 μl of ice-cold 20% trichloroacetic acid and the mixture was centrifuged. An aliquot of the supernatant was basified and extracted as described in section 1.6.1. Control incubations were carried out in which (i) substrate, (ii) cofactor and (iii) microsomes were separately omitted. All incubations were done in duplicate.

3.8 Bile duct cannulation

Male Sprague-Dawley rats (250-300 g) were rendered unconscious with distilled diethyl ether and anesthetized with an intraperitoneal (ip) injection of 1 g/kg body weight urethane in normal saline. The concentration of the urethane solution was 500 mg/ml saline. When required, additional urethane was administered ip (125 mg/kg) to maintain the anesthesia (Mudler *et al.*, 1981). The bile duct was cannulated as described by Mudler *et al.* (1981) and Shu *et al.* (1991). The procedure involves the insertion of a tube into the trachea of the rat to maintain a clear airway for respiration. A midline incision was made in the abdominal wall and the bile duct was exposed. A nick was made in the bile duct and a 2 cm length of a PE-10 tubing (i.d. 0.28 mm and o.d. 0.61 mm) was introduced into the bile duct and held in position with knotted surgical thread. The total length of the PE-10 tubing was 15 cm. The midline incision in the abdominal wall was then closed with sutures using black surgical threads. After collecting the blank bile for first 30 min, the rats were dosed ip with the drug and the bile was collected for a further period of 6h. Rats were placed under a heat lamp regulated to maintain the rectal temperature at 37°C throughout the duration of the experiment.

3.9 Derivatization techniques: general method

3.9.1 Acidic metabolites

The acidic metabolites were derivatized according to the method reported by Pearson and Sharman (1975). The residue, obtained at the end of extraction of acidic metabolites [section 3.10.4.2], was mixed with 200 µl of trifluoroacetic anhydride followed by 100 µl hexafluoroisopropanol and placed in a glass reaction vial. The reaction vial was tightly closed, the contents mixed and heated at 100°C in a metal heating module for 1h. The reaction vial was allowed to cool to room temperature before opening and the contents were evaporated *just* to dryness at room temperature under a stream of dry nitrogen.

3.9.2 Basic metabolites

The basic metabolites were derivatized using an anhydrous derivatization technique. Each dried urine extract containing the drug and basic metabolites was mixed with 100-250 µl of a mixture of acetic anhydride and dried pyridine (3:2 by vol.) and placed in a tightly closed glass tube. The tube was then heated at 60°C in a water bath for 30 min. The contents of the tube were allowed to cool to room temperature and then evaporated to dryness under a stream of dry nitrogen.

3.10 TMP experimental

3.10.1 Extraction of TMP maleate from Surmontil® tablets

Powdered Surmontil® tablets (equivalent to 300 mg base) were dissolved in 25 ml 10% HCl. The solution was extracted twice with 25 ml of diethyl ether. The ether layer was discarded and the aqueous layer was basified to pH 10-11 with 1M NaOH and extracted twice with 25 ml of diethyl ether. The combined organic layer, which contained TMP base, was evaporated using a rotary evaporator and a brownish yellow liquid was obtained. The TMP base was then converted to TMP maleate according to the following procedure.

Equimolar proportions of TMP base and maleic acid were dissolved in ethanol and stirred for 2h at room temperature and then warmed for 10 min at 40°C. The mixture was evaporated using a rotary evaporator and a yellowish brown oil was obtained. The oil was dissolved 20 ml diethyl ether and then evaporated and immediately a brown solid resulted. The resulting brown solid contained TMP maleate and was recrystallized from methanol to shining white crystals. The purity of TMP maleate salt was confirmed from its melting point (m.p.) 142.5°C [literature (Moffat et al., 1986) m.p. 140° to 144°C], ^{1}H n.m.r spectrum: ($CDCl_3$) δ: 0.95 (0.98*) [d, CH_2-CH-(**CH_3**)-CH_2-]; 2.5 (2.8) [s, -N(**CH_3**)$_2$]; 3.8-4.1 (3.1-3.9) [m, methylene protons]; 6.3 (6.15) [s, CH=CH (maleate)]; 7.1 (7.05) [aromatic protons]. [*Values in brackets indicate literature (Al-Badr, 1983) values], and from the mass spectrum: 294 (12)[$M^{+\cdot}$]; 249 (80); 234 (24); 208 (30); 193 (25), 99 (25); 84 (21) and 58 (100).

3.10.2 Dose and sample collection

A 24h urine sample from a 30 year old female patient receiving TMP, (Surmontil®) 150 mg/day, at the University of Alberta Hospitals, under the supervision of Dr. Kevin F. McKenna of the Department of Psychiatry, was collected and stored at -20°C until analysis was performed. Urine samples (24h) from two adult healthy male volunteers (RTC and MSH) were also collected after a single oral (po) dose of a 25 mg Surmontil® tablet. In *in vivo* animal metabolic studies, each rat received a single ip dose, 10 mg base/kg body weight, of TMP maleate solution in normal saline and urine samples were collected for a period of 48h unless otherwise stated and stored at -20°C until the time of analysis. Control rats received a single ip dose of 250 μl normal saline. In biliary excretion studies a single ip dose (10 mg base/kg) of TMP maleate was used and bile was collected for 8h period and stored at -20°C until analyzed.

Drug-drug interaction studies were conducted in three phases with intermittent washout periods. First, blank urine samples from drug-naive rats were collected and analyzed to determine whether endogenous compounds had retention times similar to TMP and/or its metabolites. In phase I, individual rats received a single ip dose of TMP maleate (equivalent to 5.0 mg base/kg) and 72h urine samples were collected and stored at -20°C. Before starting the second phase of study day 10 urine samples from individual rat were collected and analyzed for the presence of any residual TMP and/or its metabolites. The second phase of study was started on the 11th day of the drug-free period, in which each rat received either a single ip dose of IPR hydrochloride (equivalent to 12.5 mg base/kg) or a po dose of QND sulfate (21.7 mg base/kg),or QNN sulfate (21.7 mg base/kg) in normal saline daily for three days and urine samples were collected on the third day for 24h and analyzed to determine the possibility that a metabolite of either IPR or QND or QNN had a retention time similar to that of TMP. The third phase of study started 1h after the administration of last dose of either IPR or QND or QNN, by administering a single ip dose of TMP maleate (equivalent to 5.0 mg base/kg) and 72h urine samples were collected and stored at -20°C until analyzed.

3.10.3 Isolation of TMP and its metabolites from liver tissues

At the end of the *in vitro* studies with liver microsomes (sections 4.1.3 and 4.4.1), the protein was precipitated with 20% trichloroacetic acid and centrifuged. To an aliquot of the supernatant the internal standard IPR hydrochloride (1000 ng base), was added and the solution basified with 0.1M NaOH to pH 10.0-11.0. The basified mixture was extracted twice with 5 ml volumes of diethyl ether/methylene chloride (14:11 by vol.; solvent A) (Coutts *et al.*, 1976). After vortexing and centrifuging, the combined organic layer was dried under a stream of dry nitrogen. The dried extract was dissolved in 2 ml of 0.1M HCl and washed twice with 5 ml of diethyl ether. The acidic aqueous layer, which now contained the basic drug and metabolites, was readjusted to pH 10.0-11.0 with 0.1M NH_4OH and extracted twice with 5 ml volumes of solvent A. The combined organic layer was divided into two equal portions and both were evaporated to dryness under a stream of dry nitrogen at ambient temperature. One portion of the dried extract was derivatized by acetylation as described in section 3.9.2 and the acetylated extract was evaporated under nitrogen. Both the underivatized and acetylated residues were separately reconstituted in 50 µl of ethyl acetate and a 0.5-

2.0 µl aliquot of each of these solutions was used for GC and GC-MS analyses.

3.10.4 Isolation of TMP and metabolites from urine samples

Urine samples were first hydrolyzed enzymatically and then the drug and metabolites were extracted with a suitable organic solvent as described below.

3.10.4.1 Hydrolysis procedure

Aliquots (0.5-5 ml) of urine samples from drug-treated individual rats were separately adjusted to pH 5.0 with 0.1M sodium acetate buffer (pH 5.0) and incubated with 500 units of β-glucuronidase, type H-1 enzyme (contains both glucuronidase and arylsulfatase) per ml urine at 37°C for 20h. A 0.2% sodium chloride solution was used to prepare 500 units β-glucuronidase enzyme per 1 ml of solution. A standard incubation mixture usually contained urine, 1 vol; 0.1M sodium acetate buffer, 2 vols; and enzyme solution, 1 vol.

3.10.4.2 Extraction of acidic metabolites

The pH of the cooled incubation mixture was adjusted to 1.5-2.0 with 0.1M HCl and extracted twice with 5 ml volumes of solvent A (section 3.10.3). After centrifuging, the aqueous layer was retained for the extraction of basic metabolites, and the combined organic layer, which contained the acidic metabolites, was dried under a stream of nitrogen.

The dried urine extracts were dissolved in 0.1M NaOH and washed twice with 3 ml volumes of diethyl ether. The pH of the aqueous layer was readjusted to 1.0-2.0 with 0.1M HCl and extracted twice with 5 ml volumes of solvent A (section 3.10.3). Evaporation of the combined organic layer under nitrogen gave a residue that was derivatized by esterification (section 3.9.1). The derivatized residue was reconstituted in 1.5 ml of ethyl acetate, and an aliquot (0.5 µl) of this solution was subjected to GC-MS analysis.

3.10.4.3 Extraction of basic metabolites

To the aqueous layer (the residual urine remaining after the removal of acidic metabolites) the internal standard, (IPR hydrochloride, 1000 ng base) was added and the pH was adjusted to 10.0-11.0 with 0.1M NaOH solution. TMP and basic metabolites were then extracted by the procedure described in section 3.10.3. Derivatization of the extracted basic metabolites is described in section 3.9.2. Dried residues (underivatized and acetylated) were separately reconstituted in 50µl of ethyl acetate and 1 µl aliquot of each of these solutions was analyzed by GC-NPD and the combined GC-MS method.

3.10.5 Enzyme hydrolysis and extraction of metabolites from bile samples

3.10.5.1 Isolation of total (phase I and phase II) metabolites

Aliquots of bile samples (1 ml) from individual rats (n=3) were separately adjusted to pH 5.0 with 3N acetic acid and 2 ml of 0.1M sodium acetate buffer (pH 5.0) were added. The mixture was then incubated with 700 units of β-glucuronidase, type H-1 enzyme (contains both glucuronidase and arylsulfatase) at 37°C for 20h. At the end of the incubation period, internal standard (IPR hydrochloride, 1000 ng base) was added to the mixture and pH was adjusted to 10.0-11.0 with 0.1M NaOH solution. TMP and basic metabolites were then harvested as described in section 3.10.4.3. One portion of the dried extract was acetylated (section 3.9.2) and the underivatized and acetylated residues were separately reconstituted in 25 µl of ethyl acetate and a 1 µl aliquot of each reconstituted solution was employed for GC-NPD and GC-MS analyses.

3.10.5.2 Isolation of phase I metabolites

To a 2 ml portion of bile sample, 2 ml distilled water and 1000 ng IPR, as the internal standard, were added and the pH value was adjusted to 10.0-11.0 with 0.1M NaOH. TMP and its phase I metabolites were then exhaustively extracted three times with 7 ml volumes of solvent A (section 3.10.3).

The aqueous layer which contains phase II metabolites was reserved for further extraction. The combined organic layer was evaporated by passage of a stream of dry nitrogen at ambient temperature. The residue obtained was dissolved in 0.1M HCl acid and back extracted (section 3.9.5.1). A portion of the dried residue was derivatized by anhydrous acetylation (section 3.9.2). Both the acetylated and underivatized residues were separately reconstituted into 100 µl of ethyl acetate and aliquot (0.5-1.0 µl) of each of these solutions was used for analyses.

3.10.5.3 Hydrolysis and extraction of phase II metabolites

The residual bile (2ml) remaining after the removal of phase I metabolites, was divided into two equal portions and hydrolyzed separately with pure β-glucuronidase and sulfatase enzymes as follows.

β-*Glucuronidase hydrolysis*: To one portion (1 ml) of the bile 1 ml of distilled water was added and pH was adjusted to 6.8 with potassium phosphate buffer (pH 6.8). The mixture was incubated with 750 units of pure β-glucuronidase, type VII enzyme at 37°C for 20h.

Sulfatase hydrolysis: The second residual bile sample (1 ml) was adjusted to pH 5.0 with ammonium acetate and 1 ml of 0.1M sodium acetate buffer (pH 5.0) was added. To this mixture 42 units of aryl sulfatase, type VIII enzyme were added and the mixture was incubated for 20h at 37°C.

After hydrolysis (either with sulfatase or glucuronidase) of the residual bile, the internal standard (IPR hydrochloride, 1000 ng base) was added and the metabolites which had been conjugated were extracted by the technique described in section 3.10.5.2. The combined organic layer was dried and a portion of it was acetylated (section 3.9.2). Residues (underivatized and acetylated) were reconstituted separately in 50 µl ethyl acetate and an aliquot (1 µl) of each reconstituted sample was employed for GC-NPD and GC-MS analyses.

3.10.6 Assay procedure

The determinations were carried out on a Varian Vista 6000 GC coupled to a VG-7070E analytical organic mass spectrometer, equipped with a computerized work station. The mass spectrometer was in the electron ionization (EI) mode. The ionization energy was 50 eV and the GC-MS interface temperature was kept at 260°C, while the GC was in splitless injection mode. Chromatographic separation of TMP and its metabolites was achieved on a DB-17 (30 m x 0.25 mm. i.d.; film thickness 0.5 micron) fused silica capillary column at a column temperature of 100°C that was increased to 280°C at a rate of 16°C/min. The initial temperature was held for 2 min and the final temperature for 30 min. The injection port temperature was set at 250°C and the detector temperature was 300°C. Helium was used as carrier gas and the flow rate was 1 ml/min. The column head pressure was kept at 0.8 kg/cm^2. The assay procedure permitted the simultaneous determination of TMP and its major metabolites.

3.11 Nortrimipramine (norTMP) and 2-hydroxy trimipramine (2-OH-TMP) experimental

3.11.1 Dose and sample collection

Two groups rats (n=6 in each group) were used in this study. Individual rats in each group received either a single ip dose of norTMP maleate (5 mg base/kg) or 2-OH-TMP fumerate (5 mg base/kg) in normal saline. Urine samples were collected for a period of 72h and stored at -20°C until analyzed.

Drug-drug interaction studies involving norTMP and QND were conducted by the procedure described in section 3.10.2. In phase I, individual rats received a single ip dose of norTMP maleate (equivalent to 5 mg base/kg) and 72h urine samples were collected and stored at -20°C. The second phase of study was started on the 11th day of drug-free period, in which each rat received a single po dose of QND sulfate (21.7 mg base/kg) daily for 3 days. The third phase of study started 1h after the administration of last dose of QND, by administering a single ip dose of norTMP maleate (equivalent to 5 mg base/kg) in normal saline and 72h urine samples were collected and stored at -20°C until just prior to analysis.

3.11.2 Hydrolysis and extraction procedure

Aliquots (0.5-1.0 ml) of urine samples from treated (norTMP or 2-OH-TMP) rats were hydrolyzed enzymatically and extracted as described previously in sections 3.10.4.1 and 3.10.4.3 respectively.

3.11.3 Gas chromatography

The analyses were performed on a gas chromatograph equipped with a NPD. The chromatographic separations of norTMP and 2-OH-TMP and their metabolites were achieved on a DB-5 fused silica capillary column (30 m x 0.20 mm i.d.x 0.25µm film thickness). The oven temperature was initially maintained at 100°C for 2 min and then increased at a rate of 8°C per min to 280°C and held for 2 min.

3.12 IPR experimental

3.12.1 Treatment of animals

Rats (n=6), weighing 200-225 g, were used in this study. A single ip dose (10 mg base/kg) of IPR as its hydrochloride salt in 200-225 µl of isotonic saline was administered to individual rats. Control rats received only 200-225 µl of isotonic saline. I.p. injections were reproducible and the dose level selected did not distress the animals. Urine samples were collected over a period of 48h and stored at -20°C until analyzed. In *in vitro* studies with liver microsomes, 300 nmol (85.2.0 µg base) of IPR hydrochloride was used in each incubation mixture. In the biliary excretion study an ip dose of IPR hydrochloride (10 mg base/kg) in normal saline was used.

3.12.2 Incubation and extraction procedure

Individual urine samples (5 ml) were separately adjusted to pH 5.5 with sodium acetate buffer (pH 5.5) and incubated with 700 units of a mixture of glucuronidase and arylsulfatase (β-glucuronidase, type H-1 enzyme) per ml of urine at 37°C for a period of 20h. The pH of the cooled incubation mixture was adjusted to 2.0 with 0.1M HCl, washed with an equal volume of diethyl ether then centrifuged and the ether layer was discarded. The aqueous layer was adjusted to pH 8-8.5 with 0.5M NaOH and extracted twice with 7.5 ml volumes of a mixture of dichloromethane/isopropanol/ethyl acetate (1:1:3 by vol.). The combined organic layer was evaporated to dryness in a stream of nitrogen at ambient temperature. One portion of the dried residue was derivatized by acetylation and the resulting mixture was evaporated as described in section 3.9.2. Acetylated and underivatized dried urine extracts were separately reconstituted in 100 µl of ethyl acetate and an aliquot (2 µl) of each of these solutions was injected onto a gas chromatograph which was coupled to a mass spectrometer.

3.12.3 Isolation of IPR metabolites from liver tissues

After de-proteination of the liver tissues by addition of 500 µl of ice-cold 20% trichloroacetic acid, the resultant mixture was centrifuged at 900 g using a benchtop centrifuge. An aliquot (1 ml) of the supernatant was basified with 0.1M NaOH to pH 8.0-8.5 and IPR and metabolites were extracted as before (see section 3.12.2). A portion of the dried residue was derivatized by acetylation (section 3.9.2). The underivatized and acetylated dried residues were analyzed separately by a combined GC-MS method.

3.12.4 Extraction of IPR metabolites from rat bile sample

Aliquots of bile samples (2 ml) from individual rats (n=3) were separately adjusted to pH 5.0 with ammonium acetate and 3 ml of 0.1M sodium acetate buffer (pH 5.0) was added. The mixture was then incubated with 750 units of β-glucuronidase, type H-1 enzyme at 37°C for 20h. At the end of the incubation period the pH of the cooled incubation mixture was adjusted to 8.0-8.5 with 0.1M NaOH. Extraction and derivatization procedures for IPR and its basic metabolites are described in sections 3.12.3 and 3.9.2 respectively. The underivatized and acetylated extracts were reconstituted separately in 100 µl of ethyl acetate and a 2 µl aliquot of each reconstituted solution was employed for a combined GC-MS analysis.

3.12.5 Analytical method

Electron ionization mass spectra (EIMS) were recorded on a VG-7070E mass spectrometer interfaced with a Varian Vista 6000 gas chromatograph and linked to a PDP 11 data system. The ionization energy was 60 eV and the GC-MS interface temperature was kept at 250°C. A DB-17 fused silica capillary column (30 m x 0.25 mm. i.d.x 0.5 µm film thickness) was used to achieve the chromatographic separation of IPR and its metabolites. The initial oven temperature was maintained at 100°C for 2 min and then programmed to rise at a rate of 8°C/min to 280°C and was held at that temperature for 10 min. The injection port and detector temperatures were set at 250°C and 300°C respectively. Helium was used as carrier gas and the flow rate was 1 ml/min. The column head pressure was kept at 0.8 kg/cm^2. The analytical method provided for the simultaneous analysis of IPR and its major metabolites.

3.13 Imipramine (IMI) experimental

3.13.1 Dose and sample collection

3.13.1.1 Human study

A 24h urine sample from a 26 year old female patient, who received an oral dose of 150 mg/day (3x50 mg) of imipramine (Tofranil®), was supplied by Dr K.F. McKenna, Department of Psychiatry, University of Alberta. The urine sample was stored at -20°C until the time of the GC-MS analysis.

3.13.1.2 Biliary excretion study

After bile duct cannulation, as described in section 3.8, individual rats (n=3) received a single ip dose of 10 mg base/kg IMI hydrochloride in normal saline. Bile was collected for a period of 6h and stored at -20°C until analyzed.

3.13.2 Hydrolysis and extraction procedures

3.13.2.1 Human urine sample

A portion (500 µl) of the urine sample was hydrolyzed enzymatically by a method similar to that described in section 3.10.4.1. To the cooled incubation mixture, the internal standard (TMP maleate, 1000 ng base) was added and the pH was readjusted to 9.0-10.0 with 0.1M NaOH solution. IMI and basic metabolites were then extracted and the organic layer was dried by a similar method used for TMP in section 3.10.4.3. One portion of the dried residue was derivatized by acetylation (3.9.2) and the underivatized and acetylated residues were separately reconstituted in 50 µl of ethyl acetate and an aliquot (1 µl) of each of these solutions was used for GC and GC-MS analyses.

3.13.2.2 Rat bile sample

Aliquots of bile samples (2 ml) from individual rats were separately adjusted to pH 5.0 with ammonium acetate and 3 ml of 0.1M sodium acetate buffer (pH 5.0) were added. The mixture was then incubated with 750 units of β-glucuronidase, type H-1 enzyme at 37°C for 20h. At the end of the incubation period IMI and metabolites were extracted from the cooled incubation mixture as before (3.13.2.1). The dried urine extracts were derivatized and dried (section 3.9.2), and reconstituted in 100 µl ethyl acetate. An aliquot (1 µl) of the reconstituted solution was then subjected to GC-MS analysis.

3.13.3 Assay method

GC-MS conditions were similar to those used for TMP and its metabolites and mentioned in section 3.10.6. A DB-1 capillary column having 100% dimethyl polysiloxane crosslinked and bonded stationary phase, i.d. 0.20 mm, film thickness 0.25µm, length 30m was employed for the chromatographic separation of IMI and its metabolites

A GC-NPD was used to quantitate IMI and its major metabolites in human urine. A DB-17 fused silica capillary column (0.32 mm i.d., 0.25 µm film thickness, 30 m long) was used for the chromatographic separation of IMI, its major metabolites and the internal standard. The initial column temperature was held at 100°C for 2 min and then increased to 280°C at a rate of 8°C/min and held for 5 min.

3.14 N-*n*-butylamphetamine (NBA) experimental

3.14.1 Animals and treatment

Rats (250-450g) were housed individually in plastic metabolic cages (model E1000, Maryland Plastic Inc., New York). Water and Lab Chow (Wayne Rodent Blox, Tekland/Premier Laboratory Diets, Bartonville, Illinois) were provided *ad libitum*. The following treatments were conducted: (I) rats (n=14) received a single dose of NBA hydrochloride (0.104 mmol/kg) intraperitoneally (ip); (II) rats (n=6) were first dosed ip with QND sulfate (0.031 mmol/kg) and 1h latter with a single ip dose of NBA hydrochloride (0.104 mmol/kg; (III) rats (n=8) were administered daily a single ip dose of QND sulfate (0.031 mmol/kg) for 3 days and on the third day 1h after QND treatment, each rat received a single ip dose of NBA hydrochloride (0.104 mmol/kg). Total urine was collected for 48h and stored at -20°C until analyzed. Blank urine samples from vehicle-treated rats and from rats treated with QND only were collected and analyzed to determine whether endogenous substances or QND and/or its metabolite had a similar retention time to that of NBA.

3.14.2 Extraction procedure

Total urine volumes were measured and an aliquot (1 ml) from each sample was separately adjusted to pH 5.0 with 0.1M sodium acetate buffer and hydrolyzed with 700 units of β-glucuronidase, type H-1 enzyme at 37°C for 20h. To each hydrolyzed urine sample, internal standard [4-methoxy-NBA hydrochloride (4-OCH_3-NBA), equivalent to 500 ng base] was added. The pH of each urine sample was adjusted to 11.5-12.0 with 2M NaOH and extracted twice with 5 ml of ethyl acetate. The combined organic layer was dried under a stream of dry nitrogen gas. Each dried extract was dissolved in 1ml of 0.1M HCl and washed with 2ml of diethyl ether; the mixture was centrifuged and the organic layer discarded. The pH of the aqueous layer was readjusted to 11.5-12.0 with 2M NaOH and it was extracted twice with 5 ml volumes of diethyl ether. The combined organic extract was again dried under a stream of dry nitrogen at ambient temperature. Residues were separately reconstituted in 50 µl of ethyl acetate and a 1 µl aliquot of each of these solutions was employed for GC-NPD analysis.

3.14.3 Analytical procedure

A gas chromatograph equipped with a NPD was used to quantify NBA and its two phenolic metabolites, 4-OH-NBA and 4-OH-3-OCH_3-NBA. Chromatographic separation of NBA, 4-OH-NBA, 4-OH-3-OCH_3-NBA and the internal standard 4-OCH_3-NBA was achieved on a DB-5 fused silica capillary column (0.32 mm i.d., 0.25 μm film thickness, 30 m length). Helium was used as the carrier gas at a flow rate of 1 ml/min. The initial oven temperature was held at 100°C for 2 min, then increased at a rate of 8°C/min to 240°C and held at that temperature for 3 min. The injection port and detector temperatures were 250°C and 300°C respectively.

3.15 Phenotyping experimental

3.15.1 Subjects and method

A total of 53 adult subjects (42 female and 11 male) whose ages ranged from 21 to 63 years, took part in the study. Thirteen were healthy volunteers while the rest were patients of Drs. Kenneth Skeith and Kevin F. McKenna of the University of Alberta Hospitals. However, at the time of phenotyping none of the subjects was on any other medication. After voiding their bladder, each subject received a single oral dose of Benylin DM ® syrup at bed time (equivalent to 60 mg DM), and two urine samples (0-8h and 8-12h) were collected unless otherwise stated. The urine samples were stored at -20°C until analyzed.

3.15.2 Hydrolysis and extraction procedure

Aliquots (1 ml) of urine samples from individual subjects who received DM were separately adjusted to pH 5.0 with 0.1M sodium acetate buffer (pH 5.0) and incubated with 750 units of β-glucuronidase, type H-1 enzyme per ml urine at

37°C for 20h. A standard incubation mixture usually contained urine, 1 vol; 0.1M sodium acetate buffer, 2 vols; and enzyme solution, 1 vol.

The pH of the cooled incubation mixture was adjusted to 10.0-11.0 with 0.1M NaOH solution and extracted twice with 6 ml volumes of diethyl ether. The combined organic layer was dried under a stream of dry nitrogen. The dried extract was dissolved in 0.1M HCl (pH 2.0) and washed twice with 4 ml volumes of diethyl ether. The aqueous layer was adjusted to pH 10.0-11.0 with 0.1M NaOH and extracted twice with 6 ml volumes of diethyl ether. The combined organic layer was evaporated to dryness under a stream of dry nitrogen at ambient temperature. The dried residues were separately reconstituted in 100 µl of ethyl acetate and a 0.5 µl aliquot of these solutions was used for gas chromatographic analysis.

3.15.3 Gas chromatography

A GC-NPD was used for the analysis of DM and DR in urine extracts. Chromatographic separation of DM and DR was achieved on a DB-17 capillary column (0.32 i.d., 0.25 µm film thickness, 10 m long). The initial oven temperature was maintained at 150°C for 2 min and then increased at a rate of 16°C per min to 250°C and held there for 1 min. The injection port and detector temperatures were kept at 250°C and 300°C, respectively. The analytical method provided rapid determination of DM and DR. In fact the total analysis time was less than 7 min.

3.16 Calibration graphs

To quantitate drug and metabolites in biological matrices, standard calibration graphs were prepared using the internal standard method.

The graphs were prepared by adding varying amounts of the authentic samples of drug and metabolites (equivalent to 100-2000 ng of the free base) and a fixed amount of the internal standard (equivalent to 500 -1000 ng of the free base) to 1 ml of appropriate drug-free biological fluid. Peak area ratios (drug or metabolite/internal standard) were plotted against concentrations of drug or metabolite to produce calibration graphs that were linear over the desired concentration range. The concentration of drug and each metabolite in the biological fluid extract from drug-treated rats was determined from the equation of the straight line derived from each calibration graph. The assay methods were reproducible and the inter-assay coefficients of variation were <10%.

3.16 Statistical analyses

Where applicable the standard error of the means (S.E.M.) is represented by error bars on figures. Unless otherwise stated data were analyzed by the paired Student's t-test (α=0.05). ANOVA followed by the Newman-Keuls test was employed in section 3.14 (α=0.05) and the statistical analysis was performed by Dr. Mathew Martin-Iverson, Department of Psychiatry, University of Alberta.

4. RESULTS AND DISCUSSION

4.1 Studies conducted on TMP

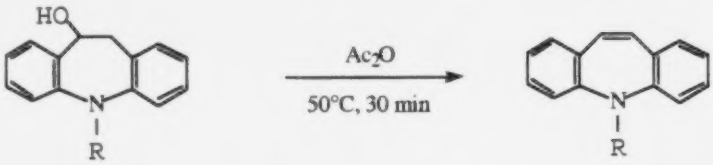
4.1.1 In vivo metabolism studies

4.1.1.1 Metabolic studies of TMP in the rat

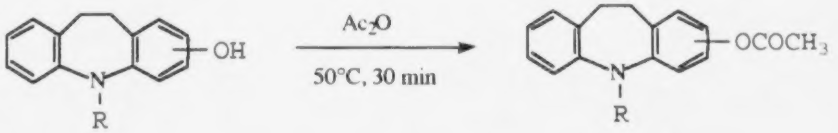
The underivatized and the acetylated metabolites of TMP were gas chromatographically separated and their chemical structures were deduced by detailed interpretations of the fragmentation patterns apparent in each electron ionization mass spectrum (EIMS). Many hydroxylated metabolites of TMP were identified. In man TMP undergoes aromatic hydroxylation in addition to other metabolic pathways, but no alicyclic hydroxylation has been observed (Maurer, 1989). It is of interest that in the present study it has been observed that in rat TMP undergoes preferential C_{10} or C_{11} hydroxylation in addition to aromatic hydroxylation.

The identities of the alicyclic and aromatic hydroxylated metabolites were readily deduced from the fragmentation patterns in the EIMS of the underivatized and acetylated samples, and by comparison of mass spectra with the spectrum of TMP itself (EIMS 1, Figure 4-3). Of particular assistance in the identification of metabolites was the observation that TMP itself and all the metabolites that retained an intact N,N-dimethyl-2-methylpropylamine [$-CH_2CH(CH_3)CH_2N(CH_3)_2$] side chain at N_5 gave rise to mass spectra that contained prominent diagnostic ions of *m/z* [M-45]$^+$, [M-60]$^+$, 99, 84 and 58, which are identified in Figure 4-4. The ion of *m/z* 58 is most often the base peak in these spectra. This observation permitted the conclusion that EIMS 1, 8, 9, 12, 13, 14, 15 and 20-26 [Figure 4-3], were all of metabolites that retain an intact N_5 side chain. Similar reasoning allowed the conclusion that mono-N-demethylated metabolites (nor-TMP and derivatives) that possessed a $CH_2CH(CH_3)CH_2NHCH_3$ side chain at N_5 may be characterized by the presence of diagnostic fragment ions of *m/z* M-31 [M-NH_2CH_3]$^+$, [M-46]$^+$, 85 and 70; these ions correspond to ions *m/z* [M-45]$^+$, [M-60]$^+$, 99 and 84 in TMP (Figure 4-4). This structural feature is clearly present in the EIMS 2, 10, 16 and 18 (Figure 4-3).

Location of a hydroxyl moiety at C_{10} or C_{11} in TMP metabolites was deduced from the observation that the mass spectra of these metabolites, before derivatization, contain an [M-18]$^+$ fragment ion due to elimination of water from the molecular ion (EIMS 6, 8 and 10; Figure 4-3). However, when derivatized by acetic anhydride, these C_{10} or C_{11} hydroxylated metabolites were dehydrated to products that contained a double bond at C_{10} (EIMS 7, 9 and 11; Figure 4-3):



In contrast, the mass spectra of aromatic hydroxylated metabolites did not contain fragment ions resulting from the elimination of water (EIMS 4, 12, 16 and 22; Figure 4-3). In addition, aromatic hydroxylated metabolites readily acetylated when reacted with acetic anhydride (EIMS 5,13,17 and 23; Figure 4-3) :



The exact position of the aromatic hydroxy, acetoxy and, in one instance (metabolite that produced EIMS 26; Figure 4-3), methoxy moieties on the tricyclic system could not be determined unequivocally from the mass spectral data. However, by analogy with the findings of Fraser *et al.*(1987) and Suckow *et al.*(1984), aromatic mono-hydroxylation most probably occurred at C_2. Further confirmation of this conclusion was obtained from the mass spectral fragmentation patterns of the authentic sample of 2-OH-TMP, which was identical with the EIMS 12, Figure 4-3.

GC traces of the underivatized and acetylated TMP metabolites are depicted in Figures 4-1 and 4-2 respectively. In figure 4-1, the peak 363 was due to endogenous cholesterol. Both the underivatized and acetylated urine extracts were analyzed under identical GC-MS conditions. GC peaks due to TMP and its metabolites were detected in both the traces. They are identified in Figure 4-1 and 4-2 by their EIMS numbers. Appropriate complete or partial structures of the isolated metabolites and their EIMS are depicted in Figure 4-3. The identities of the underivatized and acetylated metabolites of TMP were deduced by an in-depth interpretation of the EIMS obtained by scanning each GC peak. Important mass fragmentation pathways of TMP are shown in Figure 4-4. Structures of the common and major diagnostic fragment ions of all TMP metabolites are provided in Figure 4-5. Structures of urinary metabolites of TMP are shown in Figure 4-6.

Peak 354 [Fig.4-1; retention time (t_R) 14.20 min] and peak 353 [Fig.4-2; t_R 14.15 min]. Scanning of both the peaks gave similar mass spectra [EIMS 1; Figure 4-3] that contained diagnostic fragment ions of *m/z* 294 ($M^{+\cdot}$) , *m/z* 249 $[M-45]^+$, 234 $[M-60]^+$, 208, 193, 99, 84, and 58. Appropriate structures for all these fragment ions are depicted in Figure 4-4. The mass fragmentation patterns confirmed that this peak was the parent drug TMP. Its mass spectrum [EIMS 1, Figure 4-3] was identical to the published spectrum of TMP (Maurer, 1989) and with that of authentic sample of TMP. The identities of most of the TMP metabolites can be readily deduced by comparing their EIMS with that of TMP. In this study it has also been observed that the EI mass spectra of monohydroxylated TMP metabolites contain diagnostic fragment ions many of which are 16 amu higher than those in the spectrum of TMP, while equivalent fragment ions of nor-TMP are 14 amu (CH_2) less than the corresponding fragment ions of TMP.

Peak 377 [Fig.4-1; t_R 14.48 min]. This is a minor metabolite of TMP in the rat. The characteristic fragment ions in its mass spectrum [EIMS 2; Figure 4-3] are of *m/z* 280 ($M^{+\cdot}$), 249 $[M-NH_2CH_3]^+$, 234 $[M-46]^+$, 208, 195, 193, 85 and 70 [Figure 4-4]. The absence of diagnostic fragment ions of *m/z* 99, 84 and 58 showed that the N,N-dimethyl-2-methylpropylamine $[-CH_2CH(CH_3)CH_2N(CH_3)_2]$ side chain at N_5 of TMP was modified in this metabolite. The presence of ions of *m/z* 280 ($M^{+\cdot}$), 85 and 70, each 14 amu less than the corresponding diagnostic fragment ions of *m/z* 294, 99 and 84 in TMP, indicated that this peak was mono-N-demethylated-TMP (nor-TMP) [Figure 4-4]. The mass spectrum of an authentic sample of norTMP was identical to EIMS 2; Figure 4-3.

The identity of this metabolite was further supported by the mass fragmentation patterns of its acetylated derivative, [peak 681, t_R 22.43 min (Figure 4-2)]. Scanning of this peak gave EIMS 3 [Figure 4-3]. The molecular ion of *m/z* 322 ($M^{+\cdot}$) [Figure 4-3] in the spectrum was 42 amu ($COCH_2$) greater in mass than the molecular ion of nor-TMP, while other important fragment ions of *m/z* 249 $[M-CH_3NHCOCH_3]^+$(VIc), 234 (IVc) [Figure 4-5], 208 (base peak in both EIMS 2 and 3) and 193 [Figure 4-4] were identical to fragment ions in the spectrum of nor-TMP. Peak 681 is clearly the N-acetylated derivative of nor-TMP. Furthermore the EIMS 3 is identical with the published spectrum of this species (Maurer, 1989).

Peak 424 [Fig.4-1; t_R 16.01 min]. Inspection of the mass spectrum (EIMS 4; Figure 4-3) of this peak revealed its molecular ion to be of *m/z* 211 (Figure 4-3). This was consistent with a mono-oxygenated structure that lacked the N_5 side chain. It was tentatively identified as 2-OH-5H-dibenz[b,f]azepine.

The fragmentation pattern of its acetylated derivative, [peak 456, t_R 16.50 min (Figure 4-2); EIMS 5 (Figure 4-3)], confirmed this conclusion. The mass spectrum of the acetylated metabolite was virtually identical to that of 2-hydroxy-5H-dibenz[b,f]azepine, except for the presence of the expected molecular ion of *m/z* 253. Absence of dehydration during derivatization with acetic anhydride provided evidence of aromatic hydroxylation. These observations indicated that GC peak 456 was the O-acetylated derivative of 2-hydroxy-5H-dibenz[b,f]azepine. The -NH moiety of the dibenz[b,f]azepine ring was not a site for acetylation. The lone pair of electrons on the N-atom would be delocalized in this ring system making the N-atom much less basic than that of a secondary aliphatic amine. As a consequence the ring -OH group was preferentially acetylated. This deduction was further confirmed by direct comparison of EIMS 5 with the published spectrum of this compound (Maurer, 1989).

Peak 463 [Fig 4-1; t_R 17.02 min]. EIMS scanning of this peak indicated that it contained a new, though minor, metabolite of TMP. Its mass spectrum (EIMS 6; Figure 4-3) contained diagnostic fragment ions of *m/z* 227 ($M^{+\cdot}$) (Figure 4-3), 209 (Ib) and 194 (IX) (Figure 4-5). The molecular ion is 16 amu greater than that of 2-hydroxy-5H-dibenz[b,f]azepine (EIMS 4; Figure 4-3) suggestive of a dihydroxy-5H-dibenz[b,f]azepine metabolite. The fragment ion of *m/z* 209 was the result of the expulsion of a molecule of water from the molecular ion and is of particular diagnostic value. Its presence indicated that one of the hydroxy groups of this metabolite was located at C_{10} or C_{11} in the ring system. This novel metabolite is identified as 2,10- or 2,11-dihydroxy-5H-dibenz[b,f]azepine.

The identity of this metabolite was further established from the mass spectral behavior of its acetylated derivative, [peak 484, t_R 17.35 min (Figure 4-2); EIMS 7 (Figure 4-3)]. During derivatization with acetic anhydride one of the two hydroxy groups of this metabolite was involved in a dehydration reaction with the consequent introduction of a C_{10} double bond while the other hydroxy group was acetylated. This reaction is consistent with one hydroxy group being located on an alicyclic carbon (C_{10} or C_{11}) and the other on an aromatic ring. Diagnostic ions in this spectrum are of *m/z* 251 ($M^{+\cdot}$) [Fig.3] and 209 (Ib) (Figure 4-5).

Peak 432 [Fig.4-1; t_R 16.14 min]. Scanning of this GC peak revealed it to be another new metabolite of TMP.

Interpretation of its mass spectrum (EIMS 8; Figure 4-3) confirmed its identity as 10-hydroxy-TMP. Its molecular ion was of *m/z* 310 (Figure 4-3), 16 amu greater than that of TMP. The ions of *m/z* 292 $[M\text{-}H_2O]^+$ (Ij) and of *m/z* 265 $[M\text{-}45]^+$ (XII) (Figure 4-5), 99, 84 and 58 (Figure 4-4), the latter indicating an intact $-CH_2CH(CH_3)CH_2N(CH_3)_2$ side chain, are of particular diagnostic value.

The identity of this metabolite was further established from the fragmentation patterns of the product obtained by the action of acetic anhydride, [peak 359, t_R 14.20 min (Figure 4-2); EIMS 9 (Figure 4-3)], which is readily identified as 5-(3-dimethylamino-2-methylpropyl)-5H-dibenz [b,f]azepine, *i.e.* 10,11-dehydro-TMP. A molecular ion of *m/z* 292 (Figure 4-3) is consistent with this structure as is the presence of diagnostic fragment ions of *m/z* 247 $[M\text{-}45]^+$ (Ie), 232 $[M\text{-}60]^+$ (IIIc) (Figure 4-5), 99, 84 and 58 [Figure 4-4], again indicative of an intact N_5 side chain.

Peak 469 [Fig. 4-1; t_R 17.11 min]. EIMS scanning of this GC peak showed it to be another new metabolite of TMP. Its mass spectrum [EIMS 10; Figure 4-3] contained diagnostic fragment ions of *m/z* 296 ($M^{+\cdot}$) (Figure 4-3), 278 $[M\text{-}H_2O]^+$ (Ig), 265 $[M\text{-}NH_2CH_3]^+$ (XII), 247 (Ie), 232 (IIIc), 206 (IIIa) (Figure 4-5), 193, 85 and 70 (Figure 4-4). The presence of the fragment ion of *m/z* 278 suggested that the metabolite contained an alicyclic hydroxyl group. The absence of ions of *m/z* 99, 84 and 58 and the presence of fragment ions of *m/z* 265, 85 and

70 provided evidence of mono-N-demethylation of the N_5 side chain in TMP. These facts are consistent with this metabolite being the result of concurrent alicyclic hydroxylation and N-demethylation. This metabolite is tentatively identified as 10-hydroxy-nor-TMP. The mass spectrum of its acetylated derivative, [peak 705, t_R 23.15 min (Figure 4-2); EIMS 11 (Figure 4-3)] supported this conclusion.

During derivatization with acetic anhydride, C_{10}-C_{11} dehydration occurred and the N-methylamino group of the side chain was acetylated. The diagnostic fragment ions present in the mass spectrum of this metabolite are *m/z* 320 ($M^{+\cdot}$) (Figure 4-3), 247 $[M-73]^+$ (Ie), 234 (Id)) and 206 (IIIa) (Figure 4-5).

Peak 515 [Fig.4-1; t_R 18.20 min]. This GC peak's mass spectrum (EIMS 12; Figure 4-3) contained diagnostic fragment ions of *m/z* 310 ($M^{+\cdot}$) (Figure 4-3), 265 $[M-45]^+$ (VId), 250 $[M-60]^+$ (IVe), 224(IVb) and 209(Ib) (Figure 4-5), all of which are 16 amu greater in mass than the corresponding fragments ions in the TMP spectrum (EIMS 1; Figure 4-3). The presence of additional characteristic fragment ions of *m/z* 99, 84 and the base peak of *m/z* 58 (Figure 4-4), indicated that this metabolite retained an intact side chain $[-CH_2CH(CH_3)CH_2N(CH_3)_2]$. Thus, this metabolite is a ring-hydroxylated derivative of TMP. In contrast to EIMS 10 (Figure 4-3) which also contained a molecular ion of *m/z* 310, the EIMS of this peak did not contain $[M-H_2O]^+$ fragment ion of *m/z* 292. This indicated that the metabolic oxidation had taken place in an aromatic ring, and the metabolite, therefore, was a phenolic derivative of TMP. The mass spectrum of an authentic sample of 2-OH-TMP was identical to EIMS 12; Figure 4-3.

When the broad GC peak 531 (Figure 4-2; t_R 19.10 min) was repetitively scanned it was clear that it contained at least three TMP metabolites. Scanning of the early part of the peak (519) gave a mass spectrum (EIMS 13; Figure 4-3) which contained a molecular ion of *m/z* 352 (Figure 4-3), 42 amu greater than that of 2-OH-TMP. Other diagnostic fragment ions were *m/z* 307 $(M-45)^+$ (VIh), 265 (VId), 250 (IVe) [Fig.5], 99, 84 and the base peak of *m/z* 58 (Figure 4-4). Formation of a molecular ion, after derivatization with acetic anhydride, which was 42 amu greater than that of the metabolite indicated that the metabolite was phenolic. Confirmation of its identity was obtained from the observation that EIMS 13 was identical with the published spectrum of 2-acetoxy-TMP (Maurer, 1989).

Peak 533 [Fig 4-1; t_R 18.44 min]. Repetitive scannings of this intense GC peak indicated that it contained a single and abundant new metabolite of TMP. Its mass spectrum (EIMS 14; Figure 4-3) contained diagnostic fragment ions of *m/z* 308 ($M^{+\cdot}$) (Figure 4-3), 263 $[M-45]^+$ (Vc), 248 $[M-60]^+$ (VIIc) and 222 (VIIa) (Figure 4-5), all of which are 14 amu greater than the corresponding ions of TMP (EIMS 1). The formation of the fragment ions, *m/z* 263, 248, 99, 84 and 58, confirmed the presence of $-CH_2CH(CH_3)CH_2N(CH_3)_2$ moiety at N_5 of the ring system. The formation of fragment ions derived from the tricyclic ring that are 14 amu greater than the corresponding ions of TMP provided evidence of the metabolic introduction of a keto group at an alicyclic carbon (C_{10} or C_{11}) in the ring system. Since these two locations in TMP are equivalent, this metabolite is identified as 10-*oxo*-TMP.

Peak 531 (Figure 4-2; t_R 19.10 min) was also abundant in the GC scan of the acetylated extract, and its mass spectrum (EIMS 15; Figure 4-3) was virtually identical to that of EIMS 14; Figure 4-3. This observation is consistent with the proposed 10-*oxo*-TMP structure.This metabolic pathway has not been detected in man (Maurer, 1989).

Peak 567 [Fig. 4-1; t_R 19.44 min]. The mass spectrum (EIMS 16; Figure 4-3) of this GC peak contained diagnostic fragment ions of *m/z* 296 ($M^{+\cdot}$) (Figure 4-3), 265 $[M-NH_2CH_3]^+$ (VId), 250 $[M-46]^+$ (IVe), 224 (IVb), 211 (VIa) (Figure 4-5), 85 and 70 (Figure 4-4). The formation of fragment ions of *m/z* 265, 85 and 70 indicates metabolic N-demethylation. In contrast with the spectrum of the isomer 10-hydroxy-norTMP (EIMS 10; Figure 4-3), also of molecular mass of *m/z* 296, the EIMS of GC peak 567 did not contain ion of *m/z* 278 $[M-H_2O]^+$. Thus, the metabolically introduced oxygen atom is located in the aromatic ring system. Two concurrent metabolic pathways, namely, N-demethylation and aromatic hydroxylation, are obviously involved in the formation of this metabolite. The metabolite was identified as 2-OH-norTMP. EIMS 16; Figure 4-3 was identical to EIMS of an authentic sample of 2-OH-norTMP.

A metabolite of this structure would form a diacetylated derivative when reacted with acetic anhydride and peak 1300 [t_R 38.22 min (Figure 4-2)] is that diacetylated product. Its mass spectrum (EIMS 17; Figure 4-3) contained diagnostic ions of *m/z* 380 ($M^{+\cdot}$) (Figure 4-3), 84 amu greater than that of 2-hydroxy-nor-TMP (2 H atoms replaced with 2 $COCH_3$ group), 266 (IVh), 250 (IVe) and 224 (IVb) (Figure 4-5). When all these data are considered, the most likely structure for this metabolite is 2-OH-nor-TMP. EIMS 17 is identical with the published spectrum of the acetylated species (Maurer, 1989).

Peak 580 [Fig.4-1; t_R 20.05 min]. This is a new, minor metabolite of TMP. Its mass spectrum (EIMS 18; Figure 4-3) contained a molecular ion of *m/z* 294 and fragment ions of *m/z* 263 $[M-NH_2CH_3]^+$ (If), 248 $[M-46]^+$ (IIIe), 235 (VIIIa), 222 (IIIb), 209 (Ib), 194 (IX) (Figure 4-5), 85 and 70 (Figure 4-4]. The presence of fragment ions of *m/z* 263, 85 and 70 showed it to be a derivative of nor-TMP. Most of the other fragment ions in the spectrum are 2 amu less than the corresponding fragments ions in the EIMS 16; Figure 4-3. This minor metabolite appears to be the product of three metabolic reactions, namely, N-demethylation, aromatic hydroxylation and alicyclic hydroxylation of TMP followed by dehydration (Maurer and Pfleger, 1984; Testa and Jenner, 1976) to produce a double bond at C_{10}.

The mass spectral properties of the acetylated metabolite, [peak 1310, t_R 39.10 min (Figure 4-2); EIMS 19 (Figure 4-3)] are consistent with this conclusion. Diagnostic fragment ions present in this spectrum are of *m/z* 378 ($M^{+\cdot}$) (Figure 4-3), 264(IIIf), 222(IIIb) and 209(Ib) (Figure 4-5). The molecular ion is 84 amu greater than that of the unacetylated species, and this provided evidence of the presence of an aromatic hydroxy group in addition to the secondary nitrogen atom of the side chain.

Peak 725 [Fig.4-1; t_R 23.51 min]. The EIMS scanning of this GC peak revealed the existence of another new, minor metabolite of TMP. Its mass spectrum (EIMS 20; Figure 4-3) contained a molecular ion of *m/z* 324 (Figure 4-3) and fragment ions of *m/z* 279 $[M-45]^+$ (Vd), 264 $[M-60]^+$ (VIId) (Figure 4-5), 99, 84 and a base peak of *m/z* 58 (Figure 4-4), indicative of an intact N_5 side chain. Additional fragment ions of *m/z* 251 $[279-CO]^+$ (XI), 236 $[264-CO]^+$ (IIc) and 210 $[238-CO]^+$ (IIb) (Figure 4-5) may have the identities indicated, which would be consistent with this metabolite being a C_{10}- or C_{11}-*oxo*-aromatic ring-hydroxylated-TMP derivative, and most probably the C_{10} or C_{11}-ketone derivative of 2-OH-TMP.

Further evidence in favour of this identification was obtained from the mass spectral behavior of its acetylated derivative and also from related observations by Koppel *et al.*(1988). The acetylated derivative [peak 730, t_R 24.0 min (Figure 4-2); EIMS 21 (Figure 4-3)] is monoacetylated ($M^{+\cdot}$, *m/z* 366). Its mass spectrum contains other ions of diagnostic value 42 amu greater in mass than those of unacetylated species, namely 321 $[M-45]^+$ (Ve), 306 $[M-60]^+$ (VIIf), and 293 (XIII) and ions of *m/z* 279(Vd), 264(VIId), 251(XI), 238 (VIIb), 225 (Vb) (Figure 4-5), 98, 84 and 58 (Figure 4-4) which are also present in the mass spectrum of the unacetylated species.

Peak 874 [Fig.4-1; t_R 27.43 min]. The mass spectrum (EIMS 22, Figure 4-3) of this GC peak contained fragment ions of *m/z* 326 ($M^{+\cdot}$), 281 $[M-45]^+$ (VIf), 266 $[M-60]^+$ (IVg), 240 (IVd), 227 (VIb) and 225 (Ic) (Figure 4-5), all of which were 32 amu greater than equivalent fragments in the spectrum of TMP. An intact $-CH_2CH(CH_3)CH_2N(CH_3)_2$ side chain is obviously retained in this metabolite. The absence of a fragment ion peak due to elimination of water from the molecular ion, and the presence of an intact side chain indicated that metabolic oxidation of the TMP molecule had been confined to the aromatic ring(s). From these data the metabolite was concluded to be a dihydroxy-(aromatic) derivative of TMP.

Consistent with this conclusion is the formation of a diacetyl derivative, [peak 890, t_R 28.06 min (Figure 4-2); EIMS 23 (Figure 4-3)]. After derivatization with acetic anhydride the GC peak of this derivative contained diagnostically important fragment ions of *m/z* 410 ($M^{+\cdot}$) (Figure 4-3), 365 $[M-45]^+$ (VIm), 350 $[M-60]^+$ (VIk), 323 $[365-CH_2{=}C{=}O]^+$ (VIi), and other fragments of *m/z* 281 (VIf), 266

(IVg), 240 (IVd) [Fig.5], 99, 84 and the base peak of *m/z* 58 [Fig.4] which are also present in the mass spectrum of the underivatized metabolite. This metabolite is identified as 2,x-dihydroxy-TMP. The spectrum of its diacetylated derivative (EIMS 23; Figure 4-3) is identical with a published spectrum of this metabolite (Maurer, 1989).

Peak 778 [Fig.4-1; t_R 25.14 min]. This metabolite of TMP (EIMS 24; Figure 4-3) contains an intact N_5 side chain as indicated by the presence of fragment ions of 279 $[M-45]^+$ (Ih), 264 $[M-60]^+$ (IIIg) (Figure 4-5), 99, 84 and the base peak of *m/z* 58 (Figure 4-4). The molecular ion (*m/z* 324) and many fragment ions present in the mass spectrum of this metabolite (*m/z* 279, 264, 251(VIIIb), 238(IIId) and 225(Ic); Figure 4-5) are 2 amu less in mass than those of the metabolite having EIMS 22, Figure 4-3. A plausible explanation of these data is that this metabolite is the product of di-aromatic hydroxylation and mono-alicyclic hydroxylation of TMP, followed by dehydration to introduce a double bond at C_{10}.

The mass spectrum of the acetylated metabolite, [peak 815, t_R 26.15 min (Figure 4-2); EIMS 25 (Figure 4-3)], was consistent with this explanation. Its mass spectrum contained a molecular ion of *m/z* 408 (Figure 4-3) and appropriate fragment ions that permit the identification of the metabolite as 2,x-dihydroxy-9,10-dehydro-TMP.

Additional metabolites of TMP were detected only in the derivatized urine extract.

Peak 624 [Fig.4-2 ; t_R 21.14 min]. This GC peak was of a minor metabolite of TMP. The characteristic ions present in its mass spectrum (EIMS 26 ; Figure 4-3) were of *m/z* 382 ($M^{+\cdot}$) Figure 4-3], 337 $[M-45]^+$ (VIj), 322 $[M-60]^+$ (IVj), 295 (VIg), 280(IVi), 254 (IVf), 240(X) [Fig.5], 99, 84 and 58 (Figure 4-4). From this mass spectral data and by comparison with a published spectrum (Maurer, 1989) it was identified as the acetylated derivative of the hydroxy-methoxy-TMP metabolite. The formation of this metabolite provided evidence of the *in vivo* formation of a catechol derivative of TMP (cf. EIMS 22, Figure 4-3).

Peak 327 [Fig.4-2 ; t_R 13.45 min]. The mass spectrum (EIMS 27; Figure 4-3) of this peak was identical to that of 5H-dibenz[b,f]azepine, a known minor metabolite of imipramine (Maurer and Pfleger, 1984) but not of TMP. Two metabolic pathways, namely N-dealkylation of the entire side chain at N_5 of the ring system, and C_{10}- or C_{11}- alicyclic hydroxylation are involved in the formation of this metabolite. C_{10}-C_{11} dehydration may occur biochemically (Testa and Jenner, 1976; Maurer and Pfleger, 1984) or during derivatization with acetic anhydride. As this species was detected only after derivatization, it is plausible that the dehydration occurred chemically during the acetic anhydride treatment.

Peak 658 [Fig.4-2 (t_R 22.07 min); EIMS 28, Fig.4-3]. The molecular ion in this spectrum is of *m/z* 306. Other diagnostic fragment ions are of *m/z* 206 (IIIa) and 193 (Ia)(Figure 4-5). The presence of these ions suggests that this metabolite is the product of N,N-didemethylation and alicyclic hydroxylation, and that during derivatization with acetic anhydride the alicyclic hydroxy group was dehydrated with consequent introduction of a double bond at C_{10}. This metabolite is tentatively identified as N,N-didemethyl-10-hydroxy-TMP.

Peak 831[Fig.4-2 (t_R 26.37 min); EIMS 29, Fig.4-3]. The diagnostic fragment ions present in the mass spectrum of this GC peak are of *m/z* 311 ($M^{+\cdot}$), 269 $[M-CH_2=C=O]^+$ (VIe) and 227 $[M-2CH_2=C=O]^+$ (VIb) (Figure 4-5). The presence of base peak of *m/z* 227, which is 16 amu greater in mass than the base peak of *m/z* 211 in EIMS 4 and 5 (Figure 4-3), indicates that the metabolite is a dihydroxy derivative of 5H-dibenz[b,f]azepine. A lack of evidence of dehydration during derivatization suggests that both the hydroxy groups are located in an aromatic ring. From the mass spectral data, the acetylated metabolite is identified as an aromatic diacetoxy-5H-dibenz[b,f]azepine. The EIMS 29 is identical with the published spectrum of a derivatized metabolite of TMP, also deduced to possess the same structure (Maurer, 1989).

Peak 1085 [Fig.4-2; t_R 33.30 min]. Scanning of this peak revealed the existence of a new, minor metabolite of TMP. Its mass spectrum (EIMS 30; Figure 4-3) contained diagnostic ions of *m/z* 394 ($M^{+\cdot}$), 321 (Ve), 280 (VIIe) and 238 (VIIb) (Figure 4-5), all of which are consistent with the analyte being the N-acetylated, N-demethylated homolog of metabolite EIMS 21, Figure 4-3. It can be tentatively concluded from these observations that 2-OH-10-*oxo*-nor-TMP is a minor metabolite of TMP.

Peak 1200 [Fig.4-2; t_R *36.12 min].* Interpretation of the spectrum (EIMS 31; Figure 4-3) of this GC peak revealed that it is the acetylated derivative of N,N-didemethyl-2-hydroxy-TMP. Absence of dehydration during acetylation indicated that the hydroxy group was located in the aromatic ring. The diagnostic ions in the spectrum are 366 ($M^{+\cdot}$) (Figure 4-3), 266 (IVh), 224 (IVb) and 209 (Ib) (Figure 4-5). The mass spectrum is identical with the published spectrum of this metabolite (Maurer, 1989).

Peak 1240 [Fig.4-2; t_R *37.17 min].* Scanning of this peak indicated the presence of yet another new, but minor metabolite of TMP. The diagnostic fragment ions present in the mass spectrum (EIMS 32; Figure 4-3) of this metabolite are of *m/z* 364 ($M^{+\cdot}$), 264(IIIf) and 222(IIIb) (Figure 4-5), all of which are 2 amu less in mass than the corresponding fragment ions in EIMS 31. It is suggested that this metabolite is the product of three metabolic pathways, namely, aromatic hydroxylation, N,N-didemethylation and alicyclic hydroxylation. The alicyclic hydroxyl group is removed as a result of dehydration during derivatization with acetic anhydride with the introduction of a double bond at C_{10}.

Peak 531 [Fig.4-2; t_R 19.10 min]. Repetitive scanning of the latter part of this intense GC peak indicated that it contained a third metabolite of TMP. The presence of ions of *m/z* 305 $[M-45]^+$ (Ij), 290 $[M-60]^+$ (IIIh) (Figure 4-5), 99, 84 and a base peak of *m/z* 58 (Figure 4-4) in its mass spectrum (EIMS 33; Figure 4-3) again confirmed the retention of an intact N_5 side chain. Other ions present in the spectrum [*m/z* 263 (If), 248(IIIe), 2`22(IIIb) and 209(Ib); Figure 4-5] had masses 2 amu less than the corresponding ions in EIMS 13; Figure 4-3. It is believed that this metabolite was formed as a result of aromatic and alicyclic hydroxylation of TMP, followed by C_{10}-C_{11} dehydration during derivatization. From these data, it is concluded that 2,10 (or 11)-dihydroxy-TMP is a significant metabolite of TMP in the rat.

Postulated metabolic pathways

As a result of the observations and structural assignments just described, it can be concluded that four concurrent metabolic pathways have been identified for TMP in the rat:

a) alicyclic (C_{10} or C_{11}) hydroxylation.

b) aromatic hydroxylation.

c) mono- and di-N-demethylation of the dimethylaminomethylpropyl side chain ($-CH_2CH(CH_3)CH_2N(CH_3)_2$) located at N_5 of the 5H-dibenz[b,f]azepine ring.

d) N-dealkylation of the entire side chain at N_5 of the 5H-dibenz[b,f]azepine ring.

A plausible metabolic pathway of TMP could be deamination leading to 2-substituted-propionic acid derivative. Many antiinflammatory drugs, of course, are 2-arylpropionic acids (e.g. fenoprofen, ibuprofen, ketoprofen). It is pertinent to suggest that this putative propionic acid metabolite of TMP is the agent that possesses the ability of TMP administration to reduce joint pain and tenderness in arthritic patients. However, no acidic metabolite was identified in the urine extract from rats dosed with TMP, indicating that metabolic deamination does not take place.

4.1.1.2 Biliary excretion study

Biliary excretion of TMP and metabolites was studied using bile duct cannulated rats as described in section 3.8. No biliary excretion study has been reported for TMP in any species. Since structurally similar drugs IMI (Bickel and Minder, 1970; Sallee and Pollock, 1990), AMI and NT (Baier-Weber *et al.*, 1988) undergo biliary excretion in rat, it is plausible that TMP also undergoes excretion *via* bile. The biliary excretion of TMP was investigated to determine whether this was a significant route of elimination.

A single ip dose of TMP maleate was administered to the bile duct cannulated rats (250-275 g) and bile was collected for 8h as described in section 3.10.2. TMP and its phase I and phase II metabolites were extracted from the bile by the procedures stated in section 3.10.5. Both the underivatized and acetylated metabolites of TMP were gas chromatographically separated and their structures were determined by interpretations of the fragmentation patterns apparent in each EIMS, as well as by comparing with those spectra obtained from urine extract (Figure 4-3; section 4.1.1.1). The conditions used for GC-MS are described in section 3.2.2. TMP and 12 of its metabolites were identified in the bile extract. The structures of the biliary metabolites of TMP are listed in Figure 4-7.

To quantitate phase I metabolites and the metabolites that were conjugated, a GC-NPD analytical method was employed. Chromatographic separation of TMP and its metabolites was achieved on a DB-5 fused silica capillary column.

The results of the quantitative study showed that the total % recovery of TMP and metabolites in the hydrolyzed bile extract was 14.86%, of which 0.22 ± 0.032% was recovered as unchanged TMP, 1.08 ± 0.05% as norTMP, 5.88 ± 0.24% as 2-OH-TMP and 7.68 ± 1.37% as 2-OH-norTMP. Out of a total of 14.86% isolated, 13.17% was recovered as glucuronide conjugates, 0.29% as sulfatase conjugate and 1.32% was isolated as unconjugated metabolites. The major biliary metabolite of TMP in rat is 2-OH-norTMP, while the major urinary metabolite in this species is 10-*oxo*-TMP (cf. section 4.1.1.1). The 2-OH-norTMP was excreted solely as its glucuronide conjugate. It was neither excreted as sulfate conjugate nor as unconjugated species. It can thus be concluded that glucuronidation is the most important phase II metabolic reaction involved in the excretion of TMP metabolites in rat bile. Other biliary metabolites, especially 10-OH-TMP, 10-OH-norTMP as well as 10-*oxo*-2-OH-TMP, were also excreted as conjugates in appreciable amounts. Due to the non-availability of the authentic samples the quantitation of these metabolites was not possible.

It is evident from the above results that TMP and many of its metabolites undergo biliary excretion. It is plausible, therefore, that some TMP metabolites undergo enterohepatic circulation. Further studies are required to determine the extent of enterohepatic recirculation in the rat.

4.1.1.3 Metabolism of TMP in man

The total ion chromatogram of an underivatized urine extract is shown in Figure 4-9. The structures of TMP and the metabolites were deduced from mass spectral fragmentation patterns and by comparing the EIMS of individual metabolites with those derived from urine extracts (Figure 4-3; section

4.1.1.1). Comparisons were also made with the EIMS of some authentic metabolites of TMP.

In man TMP is known to undergo aromatic hydroxylation and N-dealkylation, but no alicyclic hydroxylation at the C_{10} position has so far been reported (Maurer, 1989; Cowan et al., 1988; Koppel and Tenczer, 1988, Abernethy et al., 1984). In the study now reported, in addition to unchanged TMP, 9 metabolites (Figure 4-10) were identified of which 2 are novel and are the result of alicyclic hydroxylation at the C_{10} position.

An unexpected observation was made in this study. The non-acetylated urine extract from one healthy volunteer contained one extra metabolite whose mass spectrum (Figure 4-11) contained a molecular ion of *m/z* 324. The base peak in the spectrum was of *m/z* 224 and was formed from the molecular ion by the loss of 100 amu ($-\mathrm{CH_3\dot{C}HCH_2NHCOCH_3}$) (cf. EIMS #28, Figure 4-3, section 4.1.1.1). Another diagnostic fragment ion present was of *m/z* 209. From its mass spectral fragmentation patterns this novel metabolite is tentatively characterized as N-acetyl-2-OH-N,N-didesmethyl-TMP. Its mass spectral fragmentation pattern is depicted in Figure 4-12. As this N-acetylated metabolite was detected in an underivatized urine extract, it appears that the N-acetylation occurred *in vivo* by an enzymatic reaction involving acetyl coenzyme A. This is the first reported observation of an *in vivo* acetylation of a TMP metabolite.

In vivo acetylation of primary amines (e.g. procainamide, sulfonamides, reduced clonazepam), alcohols (e.g. choline) and thiols (e.g. CoA-SH) is well known (Testa and Jenner, 1976). In a more recent study, Ganes and Midha (1987) reported an *in vivo* acetylation pathway for N-dealkylated metabolites of doxylamine in humans.

The present metabolism study of TMP has provided evidence that, in addition to the known N-dealkylation and aromatic ring hydroxylation reaction, TMP also undergoes alicyclic hydroxylation and *in vivo* N-acetylation in man. The pharmacological properties of these metabolites are unknown.

4.1.2 *In vitro* metabolism studies of TMP

4.1.2.1 Rat liver microsomes

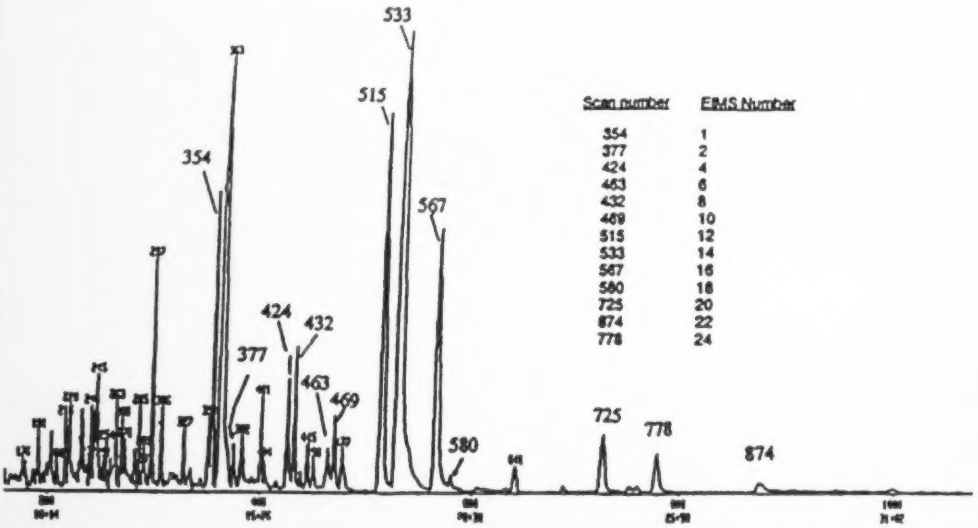
Male Sprague-Dawley rat liver microsomes were used in this study. The substrate TMP was incubated at 37°C with 1 ml fortified liver microsomes containing the required cofactors for various time intervals between 0 and 120 min.

The structures of the *in vitro* metabolites of TMP were determined by the interpretation of EIMS of individual metabolites and by comparing spectra with those of authentic metabolites and those obtained in *in vivo* metabolism studies (section 4.1.1.1). Besides unchanged TMP, 5 metabolites were characterized. The structures of *in vitro* metabolites of TMP are depicted in Figure 4-13. NorTMP was the major *in vitro* metabolite. In contrast, 10-*oxo*-TMP, the major *in vivo* metabolite of TMP in rat (section 4.1.1.1) is absent in *in vitro* studies. Demethylation and aromatic and alicyclic (C_{10} or C_{11}) hydroxylations are important *in vitro* metabolic pathways for TMP in rat. The structurally similar drug IMI also undergoes demethylation followed by aromatic and alicyclic hydroxylation when incubated with human liver microsomes (Brosen et al., 1991).

The time course of formation of different TMP metabolites is illustrated in Figures 4-14a to 4-14d. It is evident from Figures 4-14a and 4-14b that 2-OH-norTMP is formed from norTMP and not from 2-OH-TMP. Disappearance of norTMP is associated with increase in formation of 2-OH-norTMP (Figure 4-14a). In contrast, formation of 2-OH-norTMP is not associated with disappearance of 2-OH-TMP. The concentration of 2-OH-TMP remains virtually constant for the duration of the experiment with a slight decrease in concentration with time (Figure 4-14b). Therefore, the major precursor of 2-OH-norTMP is the metabolically formed norTMP. In a similar manner, the disappearance of norTMP is associated with the formation of 10-OH-norTMP. The concentrations of both 10-OH-TMP and 10-OH-norTMP increase with time and as such one metabolite cannot be the precursor of the other. Threrfore, 10-OH-norTMP is also metabolically formed from the metabolite norTMP (Figures 4-14c) and not from 10-OH-TMP (Figure 4-14d).

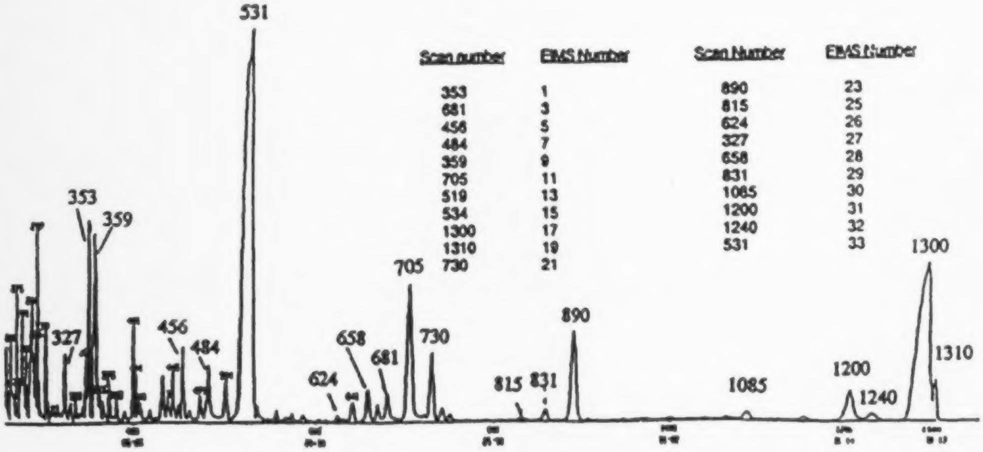
In the *in vitro* studies, it was also observed that in the absence of co-factors, most of TMP remained unchanged except for the formation of small amounts of 10-hydroxyTMP.

Therefore, co-factors are needed for the *in vitro* metabolism of TMP. This provided further evidence of the well known fact that cytochrome P450 cannot alone transfer an atom of oxygen to the substrate without the involvement of an NADPH- generating system.



Scan number	EIMS Number
354	1
377	2
424	4
463	6
432	8
469	10
515	12
533	14
567	16
580	18
725	20
874	22
778	24

Figure 4-1. Total ion current chromatogram of an underivatized urine extract from a rat dosed intraperitoneally with trimipramine.



Scan number	EIMS Number	Scan Number	EIMS Number
353	1	890	23
681	3	815	25
456	5	624	26
484	7	327	27
359	9	658	28
705	11	831	29
519	13	1085	30
534	15	1200	31
1300	17	1240	32
1310	19	531	33
730	21		

Figure 4-2. Total ion current chromatogram of an acetylated urine extract from a rat dosed intraperitoneally with trimipramine.

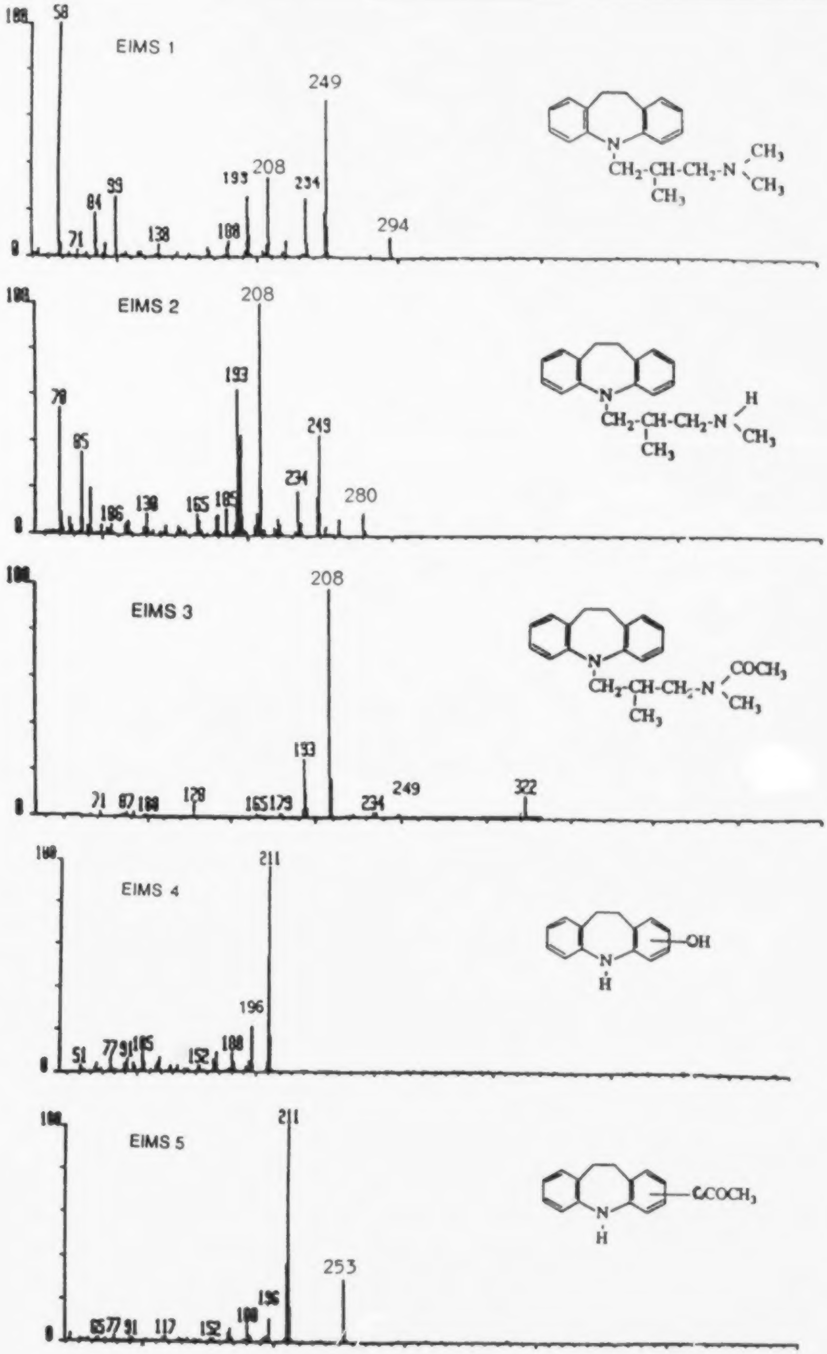


Figure 4-3. Mass spectra and structures of TMP and its underivatized and acetylated metabolites.

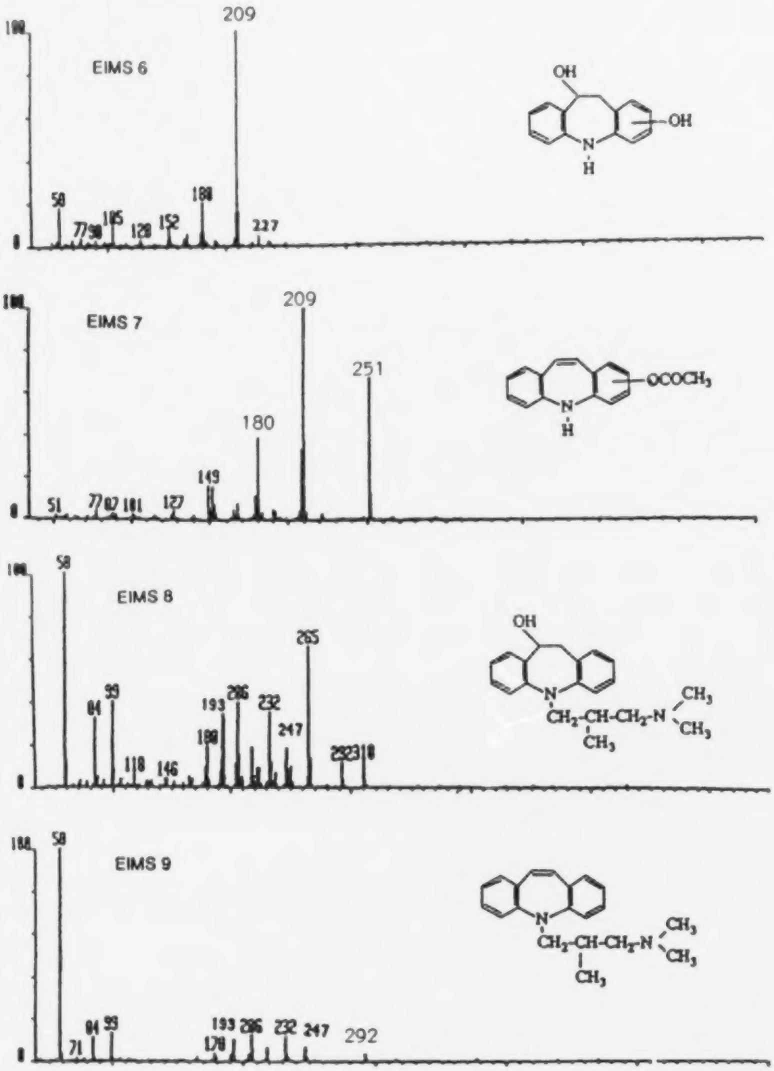


Figure 4-3. Continued

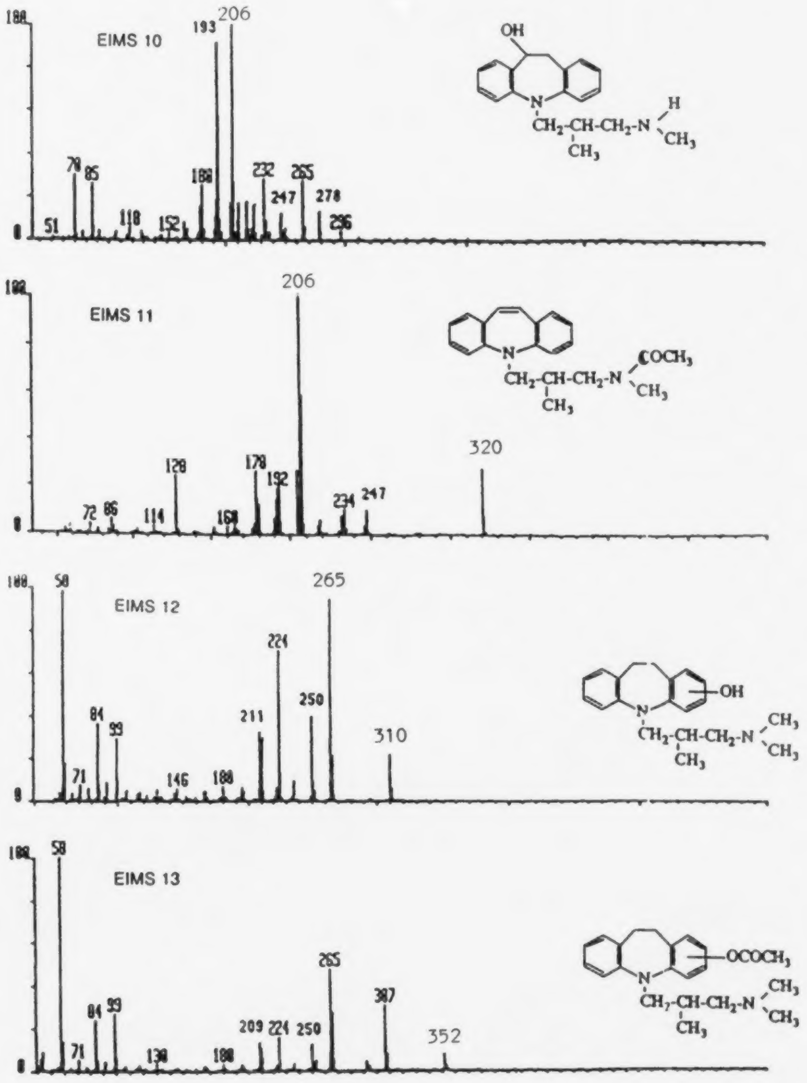


Figure 4-3. Continued

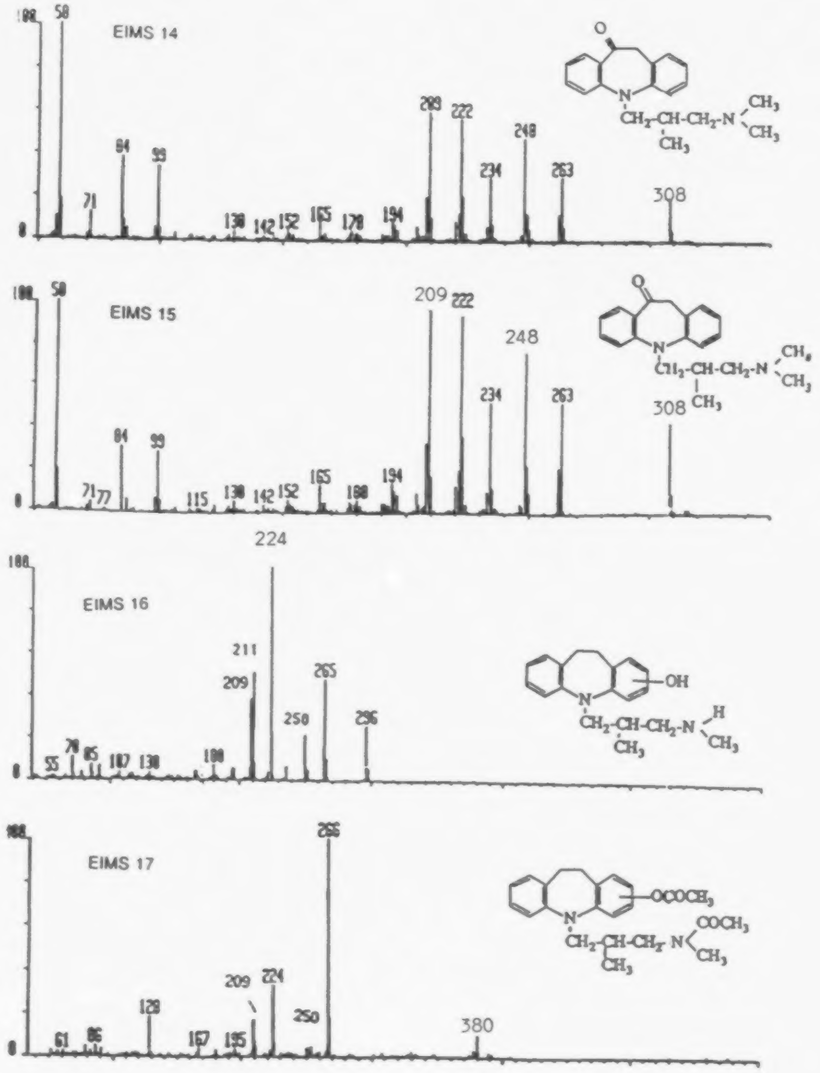


Figure 4-3. Continued

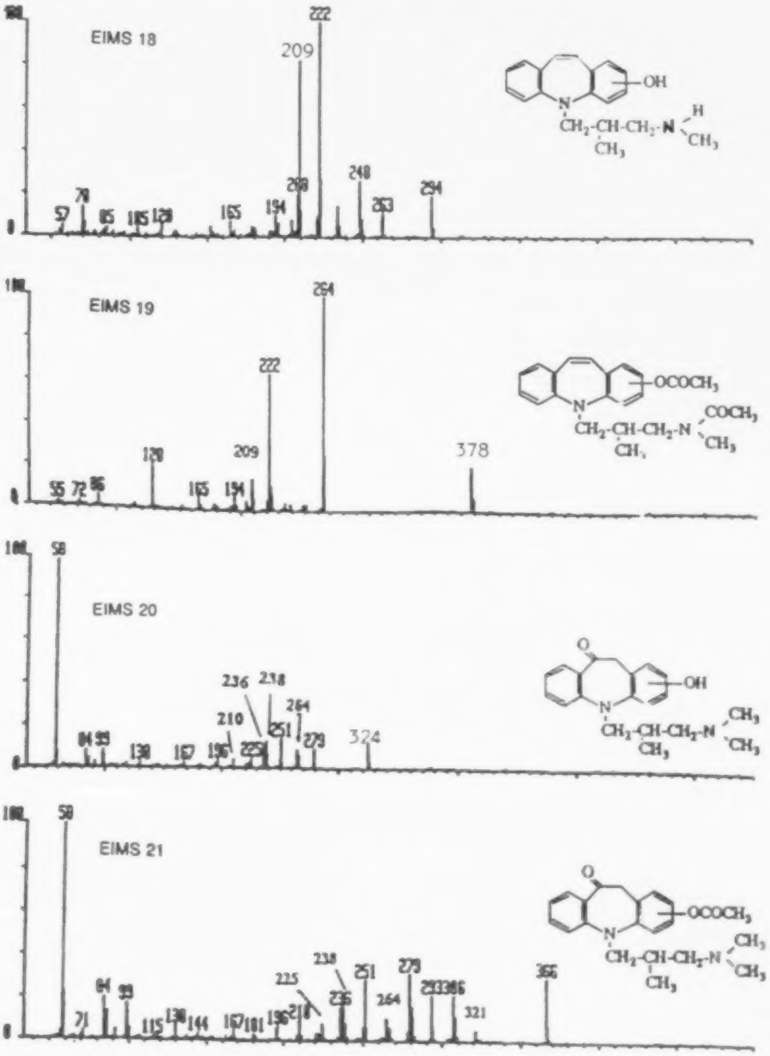


Figure 4-3. Continued

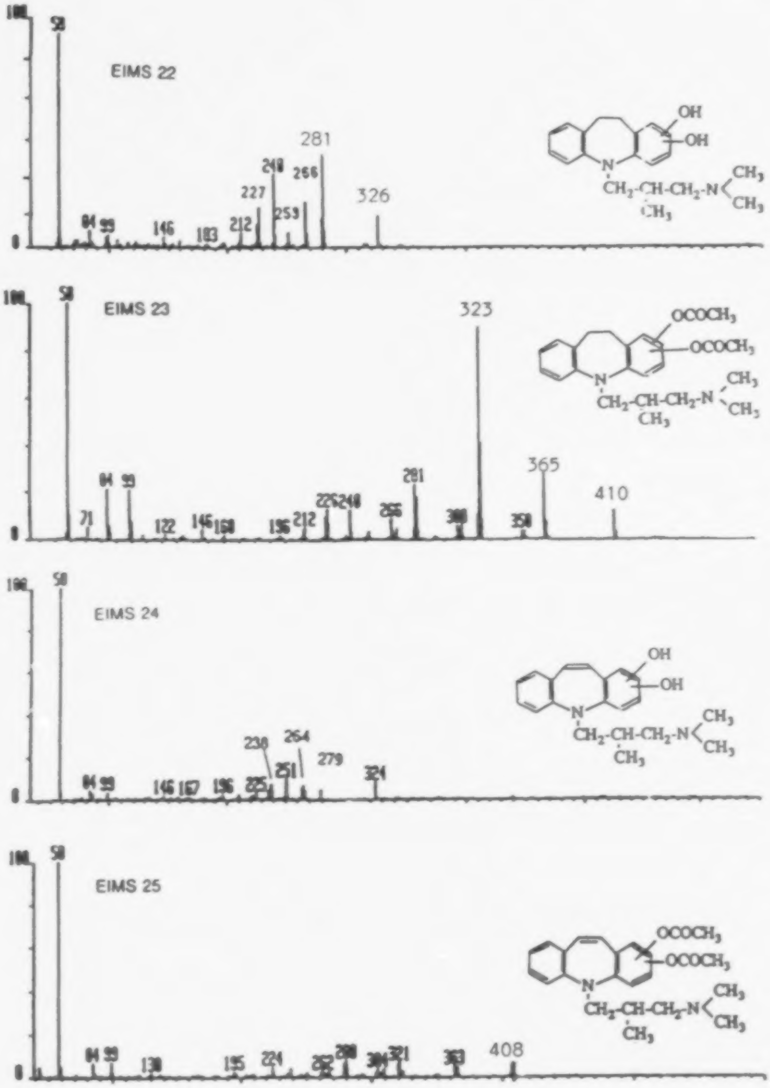


Figure 4-3. Continued

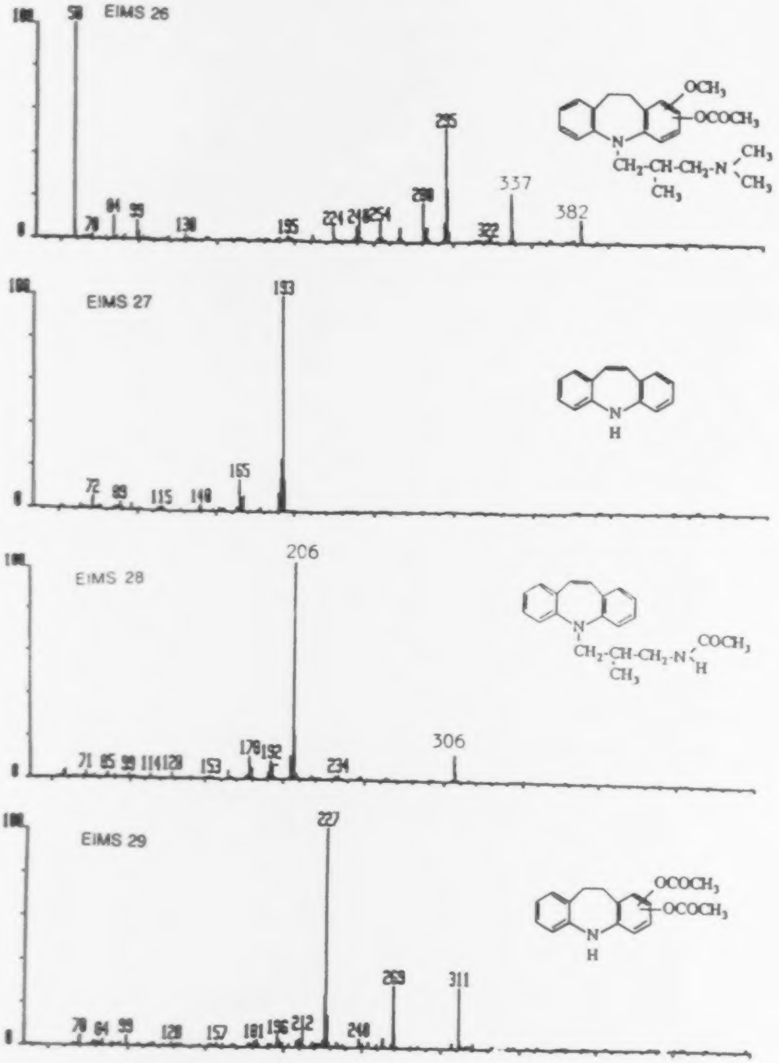


Figure 4-3. Continued

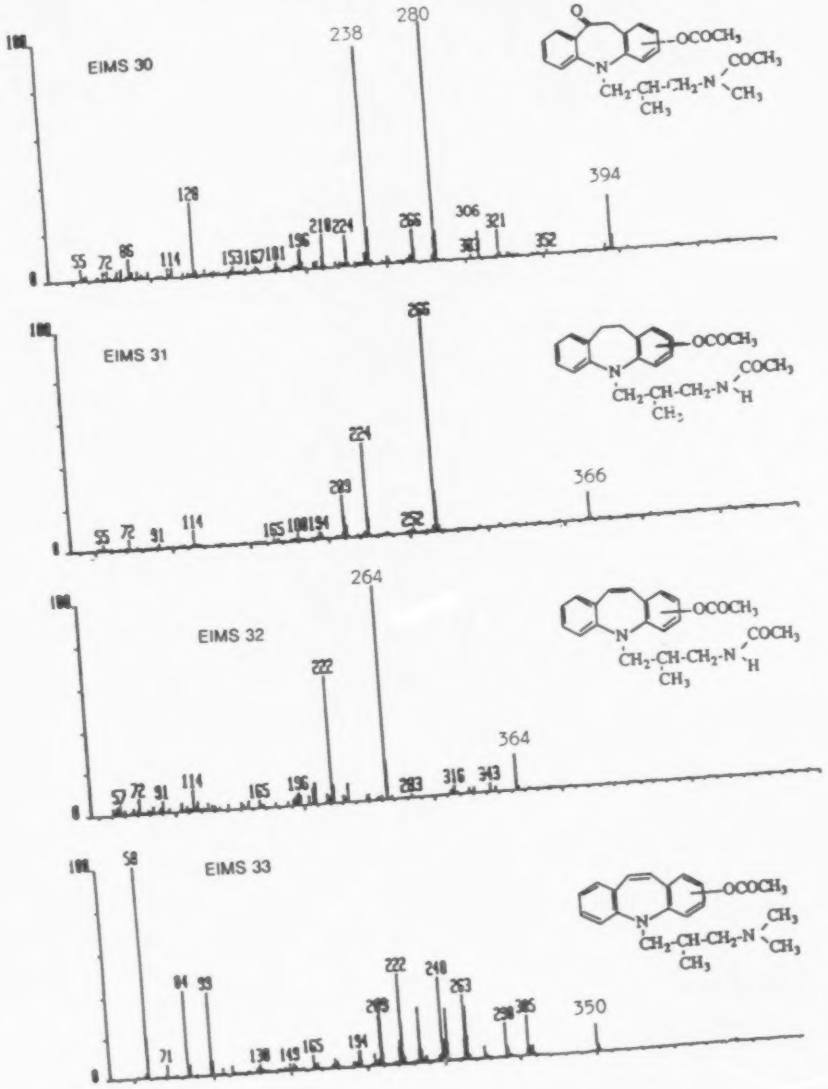


Figure 4-3. Continued.

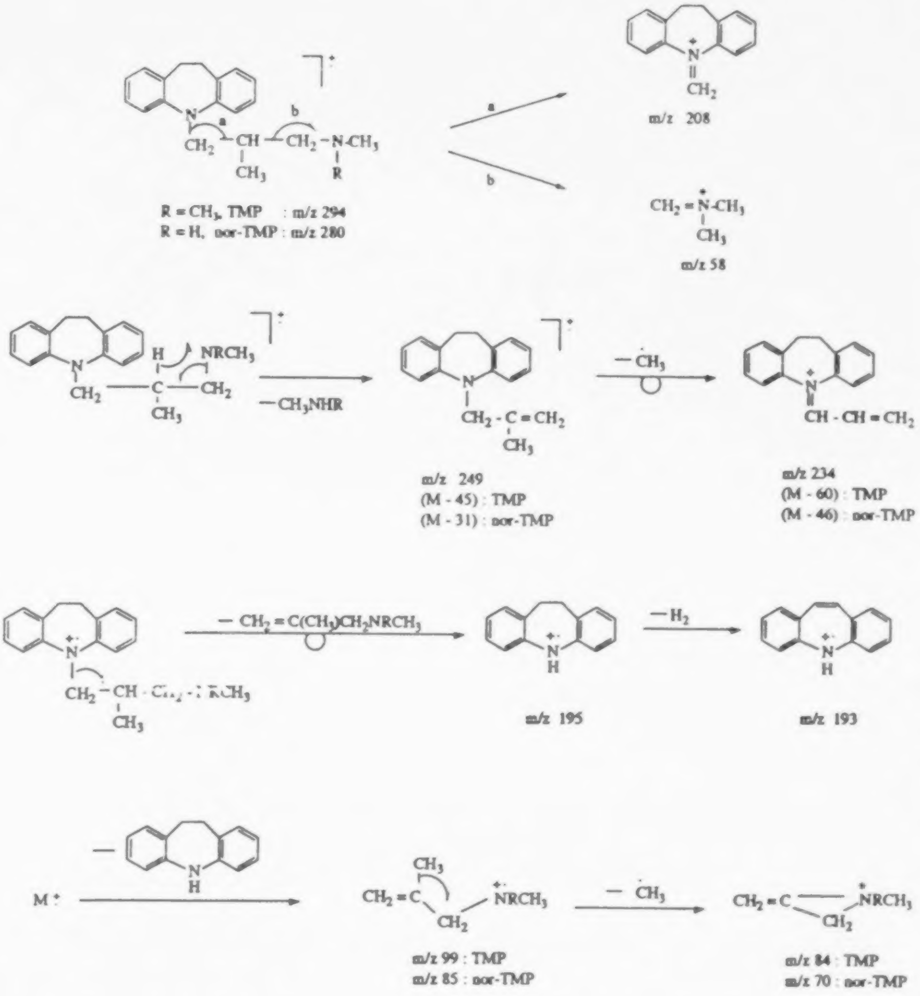
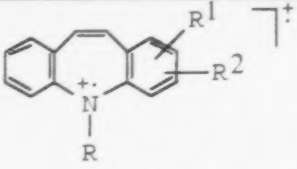

a
b
R = CH3, TMP : m/z 294
R = H, nor-TMP : m/z 280
m/z 208
m/z 58
− CH3NHR
− CH3
m/z 249
(M - 45) : TMP
(M - 31) : nor-TMP
m/z 234
(M - 60) : TMP
(M - 46) : nor-TMP
− CH2 = C(CH3)CH2NRCH3
− H2
m/z 195
m/z 193
M+·
m/z 99 : TMP
m/z 85 : nor-TMP
− CH3
m/z 84 : TMP
m/z 70 : nor-TMP

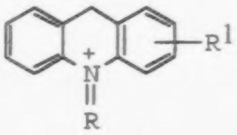
Figure 4-4. Mass fragmentation pathways of TMP and norTMP.



(I)

	R	R^1	R^2	m/z
a.	H	H	H	193
b.	H	H	OH	209
c.	H	OH	OH	225
d.	$CH_2\overset{+}{C}HCH_3$	H	H	234
e.	$CH_2C(CH_3)=CH_2$	H	H	247
f.	$CH_2C(CH_3)=CH_2$	OH	H	263
g.	$CH_2CH(CH_3)CH_2NHCH_3$	H	H	278
h.	$CH_2C(CH_3)=CH_2$	OH	OH	279
i.	$CH_2CH(CH_3)CH_2N(CH_3)_2$	H	H	292
j.	$CH_2C(CH_3)=CH_2$	$OCOCH_3$	H	305

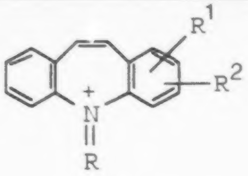
[Odd electron ions except for structure Id]



(II)

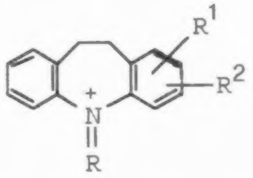
	R	R^1	m/z
a.	CH_2	H	194
b.	CH_2	OH	210
c.	$CHCH=CH_2$	OH	236

Figure 4-5. Structures of major diagnostic fragment ions of TMP and its metabolites.



(III)

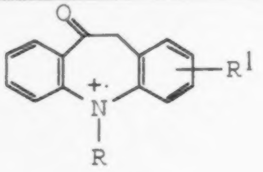
	R	R^1	R^2	m/z
a.	CH_2	H	H	206
b.	CH_2	OH	H	222
c.	$CHCH=CH_2$	H	H	232
d.	CH_2	OH	OH	238
e.	$CHCH=CH_2$	OH	H	248
f.	CH_2	$OCOCH_3$	H	264
g.	$CHCH=CH_2$	OH	OH	264
h.	$CHCH=CH_2$	$OCOCH_3$	H	290



(IV)

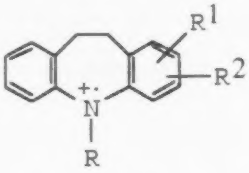
	R	R^1	R^2	m/z
a.	CH_2	H	H	208
b.	CH_2	OH	H	224
c.	$CHCH=CH_2$	H	H	234
d.	CH_2	OH	OH	240
e.	$CHCH=CH_2$	OH	H	250
f.	CH_2	OH	OCH_3	254
g.	$CHCH=CH_2$	OH	OH	266
h.	CH_2	$OCOCH_3$	H	266
i.	$CHCH=CH_2$	OH	OCH_3	280
j.	$CHCH=CH_2$	$OCOCH_3$	OCH_3	322

Figure 4-5. Continued.



(V)

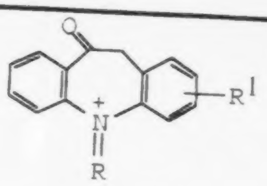
	R	R^1	*m/z*
a.	H	H	209
b.	H	OH	225
c.	$CH_2C(CH_3)=CH_2$	H	263
d.	$CH_2C(CH_3)=CH_2$	OH	279
e.	$CH_2C(CH_3)=CH_2$	$OCOCH_3$	321



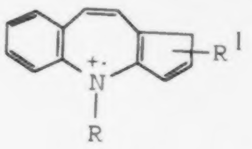
(VI)

	R	R^1	R^2	*m/z*
a.	H	OH	H	211
b.	H	OH	OH	227
c.	$CH_2C(CH_3)=CH_2$	H	H	249
d.	$CH_2C(CH_3)=CH_2$	OH	H	265
e.	H	OH	$OCOCH_3$	269
f.	$CH_2C(CH_3)=CH_2$	OH	OH	281
g.	$CH_2C(CH_3)=CH_2$	OH	OCH_3	295
h.	$CH_2C(CH_3)=CH_2$	$OCOCH_3$	H	307
i.	$CH_2C(CH_3)=CH_2$	OH	$OCOCH_3$	323
j.	$CH_2C(CH_3)=CH_2$	$OCOCH_3$	OCH_3	337
k.	$CHCH=CH_2$	$OCOCH_3$	$OCOCH_3$	350
m.	$CH_2C(CH_3)=CH_2$	$OCOCH_3$	$OCOCH_3$	365

Figure 4-5. Continued.



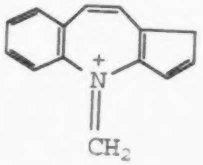
(VII)



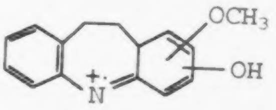
(VIII)

	R	R^1	*m/z*
a.	CH_2	H	222
b.	CH_2	OH	238
c.	$CHCH=CH_2$	H	248
d.	$CHCH=CH_2$	OH	264
e.	CH_2	$OCOCH_3$	280
f.	$CHCH=CH_2$	$OCOCH_3$	306

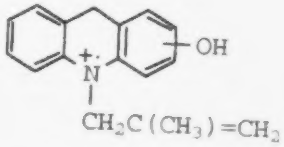
	R	R^1	*m/z*
a.	$CH_2C(CH_3)=CH_2$	H	235
b.	$CH_2C(CH_3)=CH_2$	OH	251



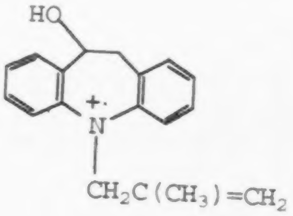
m/z 194 (IX)



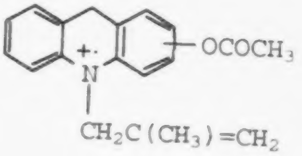
m/z 240 (X)


OH
+·
N
CH₂C(CH₃)=CH₂

m/z 251 (XI)


HO
+·
N
CH₂C(CH₃)=CH₂

m/z 265 (XII)


OCOCH₃
+·
N
CH₂C(CH₃)=CH₂

m/z 293 (XIII)

Figure 4-5. Continued.

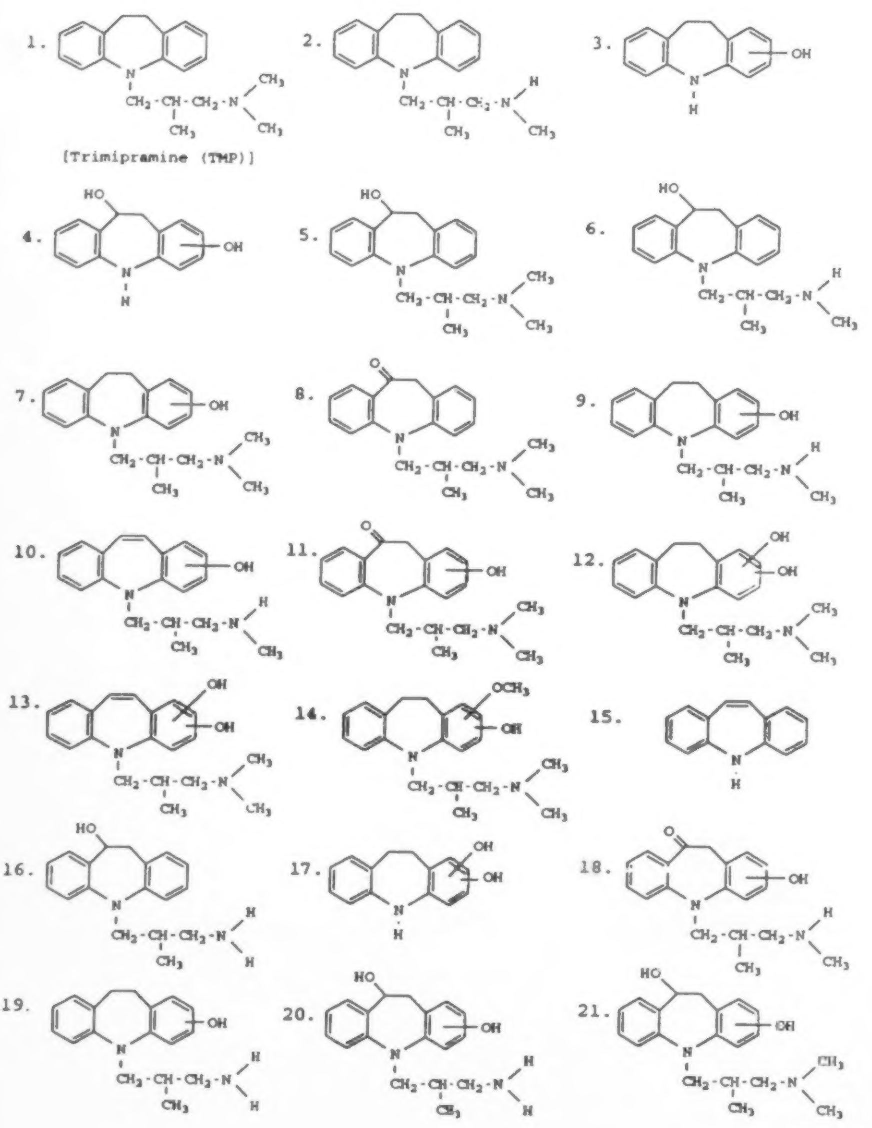

1.
[Trimipramine (TMP)]
2.
3.
4.
5.
6.
7.
8.
9.
10.
11.
12.
13.
14.
15.
16.
17.
18.
19.
20.
21.

Figure 4-6. Structures of urinary metabolites of TMP in the rat.

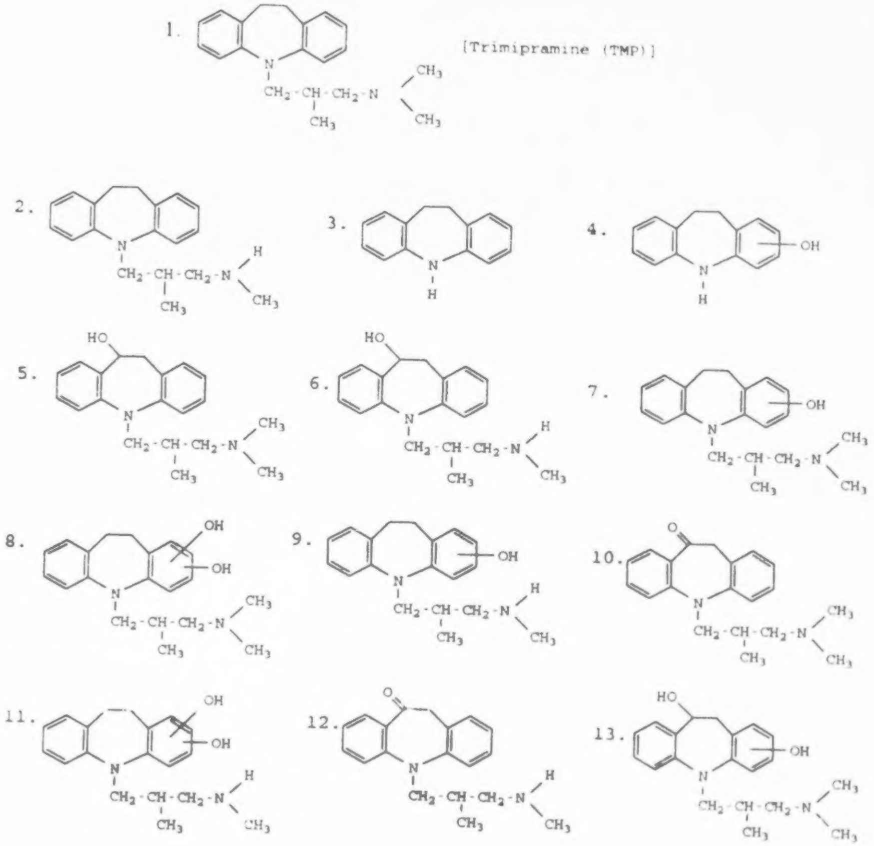
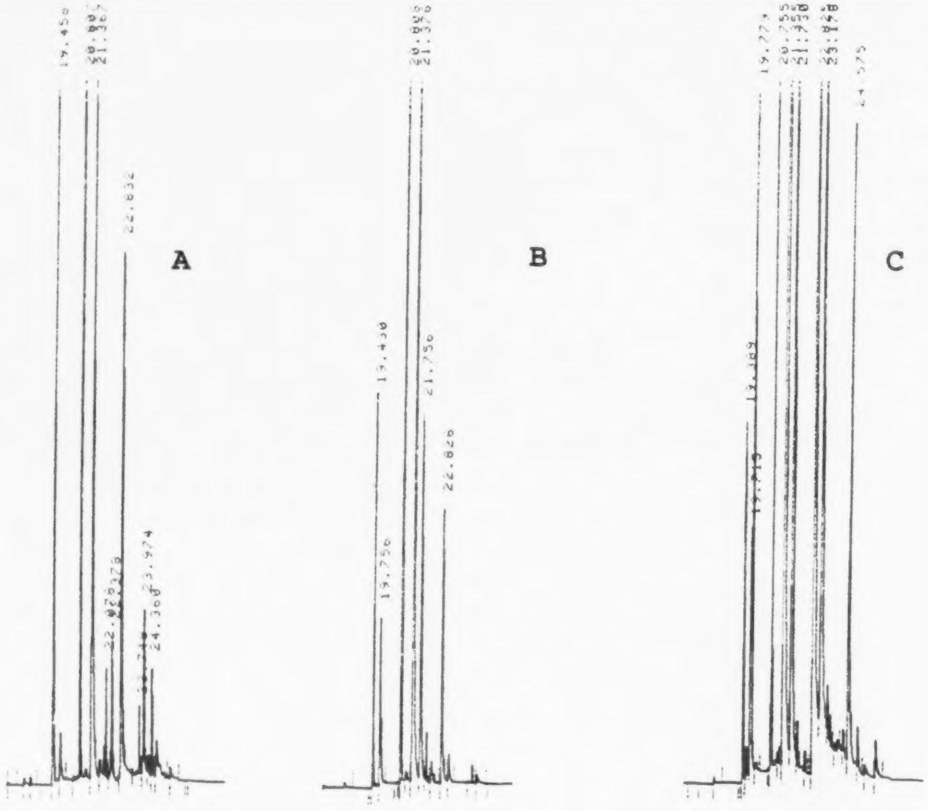

1.
[Trimipramine (TMP)]
2.
3.
4.
5.
6.
7.
8.
9.
10.
11.
12.
13.

Figure 4-7. Structures of the biliary metabolites of TMP in the rat.

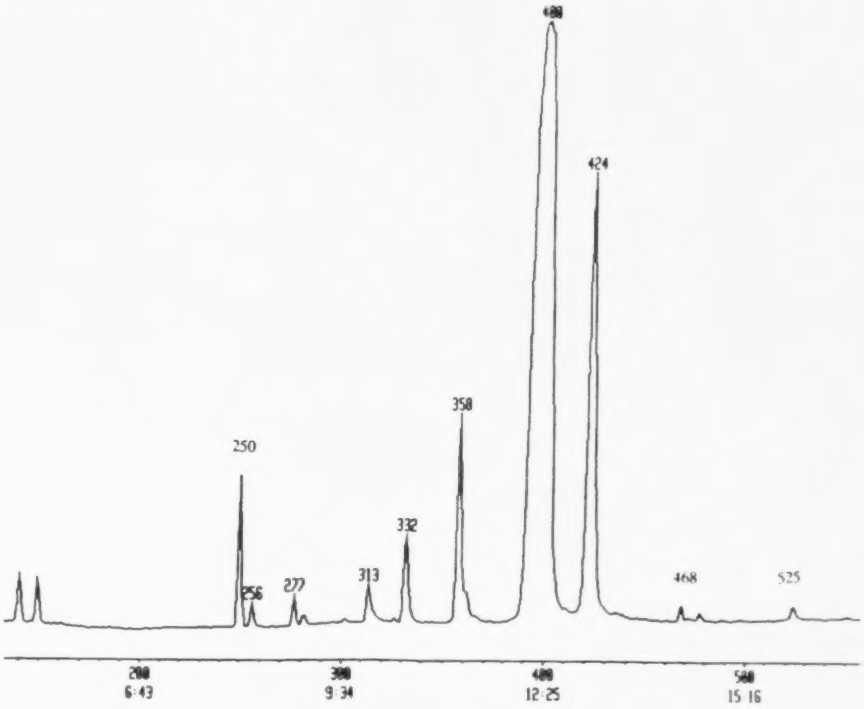


Peak identities:

Retention time (min)	*Metabolite*
19.4	TMP
19.7	norTMP
20.8	IPR (I.S)
21.3	10-OH-TMP
21.7	10-OH-norTMP
22.8	2-OH-TMP
23.1	10-oxo-TMP
24.5	2-OH-norTMP

Figure 4-8. Typical GC traces of bile extracts from rats dosed intraperitoneally with trimipramine.

A: Unhydrolyzed bile extract.
B: Sulfatase-hydrolyzed bile extract.
C: β-Glucuronidase-hydrolyzed bile extract.



Strcture # (Figure 4-10)	Scan #	Metabolite
1	250	TMP
2	256	10,11-dehydroTMP
3	277	norTMP
4	313	2-OH-IDB
5	332	10-OH-TMP
6	358	10-OH-norTMP
7	400	2-OH-TMP
8	424	2-OH-norTMP
9	468	2-OH-N,N-didesmethyl-TMP
10	525	2,3-Dihydroxy-TMP

Figure 4-9. Total ion chromatogram of an underivatized urine extract from man dosed orally with trimipramine.

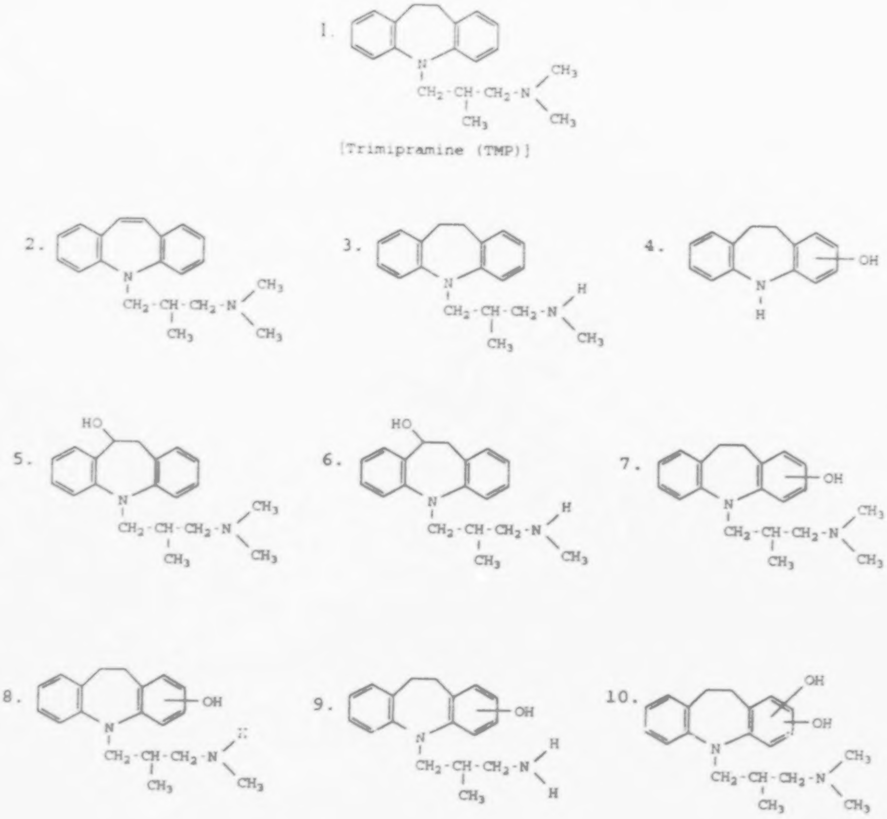

1.
N
CH₂-CH-CH₂-N
CH₃
CH₃
CH₃
[Trimipramine (TMP)]
2.
3.
4.
5.
6.
7.
8.
9.
10.
HO
OH
H

Figure 4-10. Structures of urinary metabolites of TMP in man.

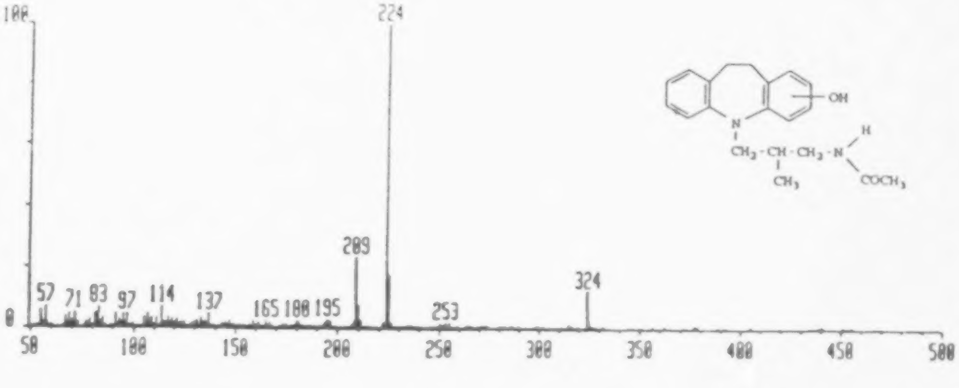


Figure 4-11. Electron ionization mass spectrum of N-acetyl-2-OH-N,N-didesmethyl-TMP.

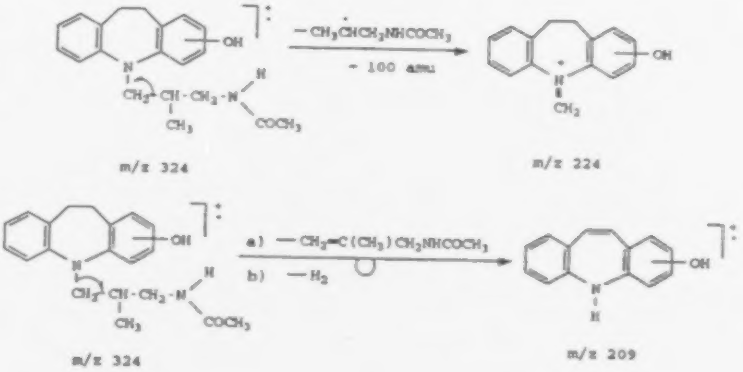
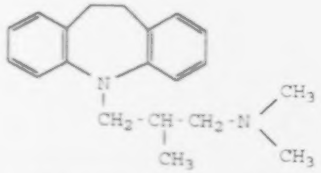

OH
— CH3ĊHCH2NHCOCH3
- 100 amu
N
CH2 CH - CH2 - N
H
CH3
COCH3
m/z 324
N
CH2
m/z 224
a) — CH2=C(CH3)CH2NHCOCH3
b) — H2
m/z 324
N
H
m/z 209

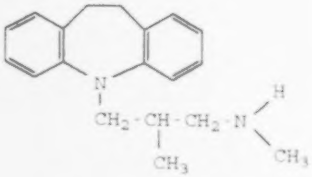
Figure 4-12. Mass fragmentation pathway of N-acetyl-2-OH-N,N-didesmethyl-TMP.

1.

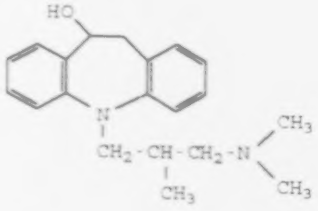

N
CH₂-CH-CH₂-N
CH₃
CH₃
CH₃

[Trimipramine (TMP)]

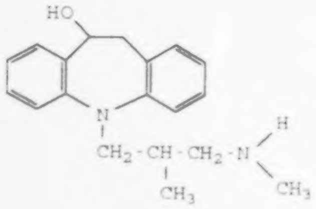
2.


N
CH₂-CH-CH₂-N
H
CH₃
CH₃

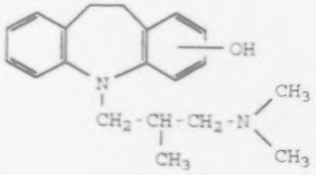
3.


HO
N
CH₂-CH-CH₂-N
CH₃
CH₃
CH₃

4.


HO
N
CH₂-CH-CH₂-N
H
CH₃
CH₃

5.


OH
N
CH₂-CH-CH₂-N
CH₃
CH₃
CH₃

6.

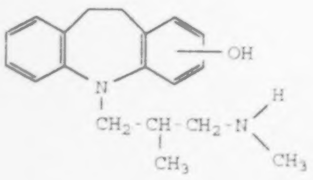

OH
N
CH₂-CH-CH₂-N
H
CH₃
CH₃

Figure 4-13. Structures of *in vitro* metabolites of TMP in the rat.

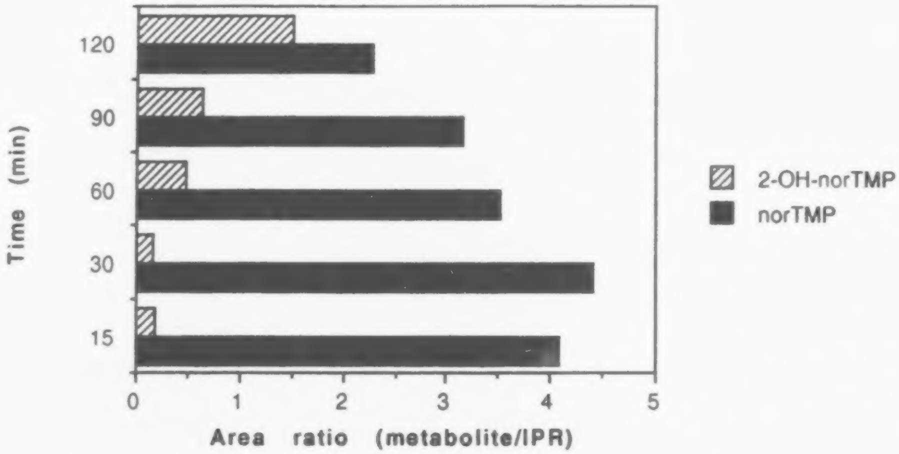


Figure 4-14a. Kinetics of formation of 2-OH-norTMP relative to norTMP from TMP in rat liver microsomes. Metabolites are assayed gas chromatographically at different time intervals.

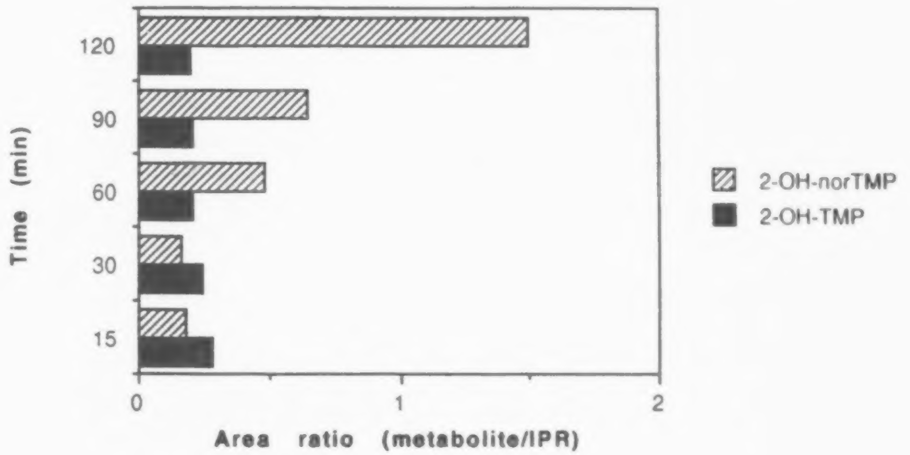


Figure 4-14b. Kinetics of formation of 2-OH-norTMP relative to 2-OH-TMP from TMP in rat liver microsomes.

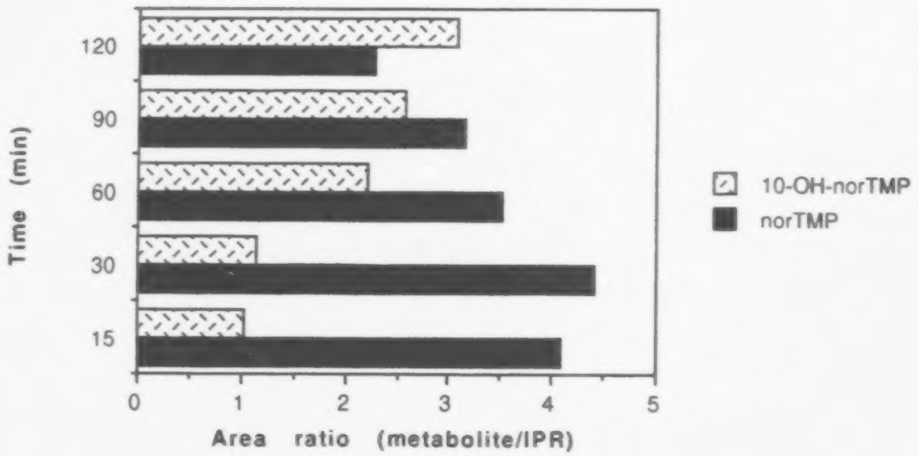


Figure 4-14C. Kinetics of formation of 10-OH-norTMP relative to norTMP from TMP in rat liver microsomes.

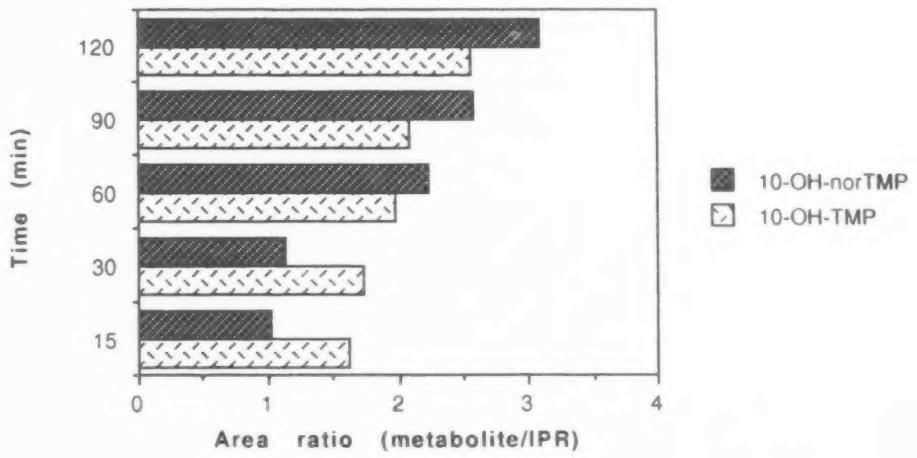


Figure 4-14d. Kinetics of formation of 10-OH-norTMP relative to 10-OH-TMP from TMP in rat liver microsomes.

4.1.3 Drug-drug interaction studies

4.1.3.1 Effects of IPR on the metabolism of TMP [1]

The second generation TCA IPR was introduced clinically in 1967, but its mechanism of action is still not known. What is known of IPR is its ability to inhibit metabolic ring oxidation of aromatic compounds (*e.g.* amphetamine) (Freeman and Sulser, 1972; Fuller and Hemrick-Luecke, 1980; Steranka, 1982), and this has lead researchers to study in more detail the effects of IPR on the metabolism of different substrates.

Li *et al.*(1988) demonstrated that IPR is an inhibitor of rat phosphatidylinositol synthetase, but the therapeutic significance of this observation is not known. Additional studies by Green *et al.*(1989) revealed that IPR was one of various compounds that partially protected mouse brain monoamine oxidase from irreversible inhibition by phenelzine. It was also demonstrated that IPR significantly reduces brain and plasma concentrations of tyrosine (Edwards *et al.*, 1988).

Of more relevance to the current study are the demonstrations in rats that IPR shares with IMI and its analogs the ability to enhance many central actions of amphetamine (Gluckman and Baum, 1969; Miller *et al.*, 1970). This enhancement of central activity in rats has been shown to be the result of inhibition by IPR of metabolic p-hydroxylation of amphetamine, leading to greatly increased tissue levels of this CNS stimulant (Freeman and Sulser, 1972; Fuller and Hemrick-Luecke, 1980; Steranka, 1982). In *in vitro* studies with 100,000 g rat liver microsomes it has also been shown that IPR acts as a competitive inhibitor of ring hydroxylation of β-phenylethylamine (PEA) to *p*-tyramine (Mosnaim *et al.*, 1989). These studies all indicate that IPR may be a potent inhibitor of metabolic aromatic hydroxylation of various substrates in rat.

---

[1] This chapter is a continuation of studies by the Neurochemical Research Unit, University of Alberta, on drug/drug interactions involving antidepressants.

In one recent study Hegadoren *et al.*(1990) demonstrated that pretreatment of rats with IPR greatly decreased the metabolic N-deethylation of the anorexiant fenfluramine to norfenfluramine, a pharmacologically active metabolite. This was the first indication that IPR inhibits metabolic N-dealkylation.

It is of interest that IPR is an arylalkyl tertiary amine and both aromatic ring hydroxylation and side chain dealkylation are likely biotransformation pathways. A further study on the metabolism of IPR in the rat (section 4.4.1) suggested that IPR inhibited its own metabolic aromatic hydroxylation. Many other antidepressant drugs, including IMI and its congeners, are metabolized to ring-hydroxylated and N-dealkylated products. The prior or concurrent administration of IPR with these and other antidepressants may constitute a significant drug/drug interaction that greatly influences the relative importance of prominent antidepressant metabolic pathways, and the pharmacological profiles of both IPR and the drug with which it is coadministered.

Drug metabolic ring-hydroxylation and N-dealkylation reactions are generally catalyzed by cytochrome P450 isozymes (Brosen, 1990). In order to learn more about these isozymes and ways in which they can be inhibited, a study in rats of the effect of preadministration of IPR on the metabolism of the IMI congener TMP was undertaken. A detailed account of the metabolism of TMP in the rat is described in section 4.1.1.1. The identified dominant biotransformation pathways of TMP were aromatic hydroxylation, aliphatic hydroxylation, both with and without N-demethylation. It was of interest to determine whether one or more of these pathways was/were significantly inhibited by prior administration of IPR, before determining which cytochrome P450 isozymes were associated with each metabolic pathway. Therefore, a study was designed to delineate the effects of IPR on the metabolism of TMP in the rat. The structures of TMP and relevant metabolites are provided in Figure 4-6; section 4.1.1.1.

The study was designed and conducted as described in section 3.10.2.

Urine samples were hydrolyzed enzymatically and TMP and metabolites were extracted as described in sections 3.10.4.1 and 3.10.4.3. Fluoxetine (FXN) hydrochloride (equivalent to 1000 ng of the free base) was used as the internal standard. Quantitation of TMP metabolites was achieved using a gas chromatograph equipped with a NPD. Calibration graphs for TMP, norTMP, 2-OH-TMP and 2-OH-norTMP were prepared by the procedure described in section 3.15.

GC traces of urine extracts from rats treated with TMP and IPR+TMP are shown in Figure 4-15. In GC traces A and B peaks corresponding to t_R 9.4, 11.3 and 18.8 min were due to endogenous substances. In trace B the peaks corresponding to t_R 17.6, 23.9,24.3 and 25.1 min were due to IPR metabolites. It was confirmed that extracts of control urines contained no GC peaks that corresponded to those of TMP, its metabolites and the internal standard. Analysis of day-10 urine samples revealed the absence of either TMP or any of its metabolites.

The most abundant urinary metabolites isolated from male Sprague-Dawley rats that had received TMP were 2-OH-TMP, 2-OH-*nor*TMP, 10-*oxo*-TMP, and 2-OH-10-*oxo*-TMP, indicative of the dominance of alicyclic oxidation (10-*oxo* formation) and aromatic hydroxylation (2-hydroxylation) (section 4.1.1.1). The extent of N-demethylation, revealed mainly by the formation of 2-OH-*nor*TMP, was less important than ring oxidation.

In the present study, the preadministration of IPR to rats that subsequently received TMP clearly affected both cyclic oxidation reactions. Visual inspection of the resulting GC trace (Figure 4-15B) revealed a virtual absence of peaks corresponding to 10-*oxo*-TMP and 2-OH-10-*oxo*-TMP. From this, it was immediately apparent that the IPR pretreatment had resulted in almost complete inhibition of alicyclic 10-oxidation of the TMP nucleus. In addition, the relative quantities of 2-OH-TMP and 2-OH-*nor*TMP were significantly altered from those produced in the absence of IPR. The quantitative results of the analysis revealed that 0.89 ± 0.11% of the administered dose was recovered as unchanged TMP, 0.83 ± 0.13% as norTMP, 19.37 ± 2.87% as 2-OH-TMP and 25.77 ± 4.36% as 2-OH-norTMP from rats that had received only TMP. Pretreatment of rats with three doses of IPR prior to TMP administration altered the recoveries of TMP, norTMP, 2-OH-TMP and 2-OH-norTMP to 1.42 ± 0.13%, 0.67 ± 0.11%, 5.7 ± 0.66% and 31.77 ± 3.14%, respectively. Figure 4-16 shows the urinary concentrations of TMP and its two major metabolites before (Phase 1) and after (Phase 2) IPR treatment.

Thus, pretreatment with IPR resulted in a statistically significant large decrease in the amount of urinary 2-OH-TMP. The recoveries of norTMP were not altered significantly by IPR pretreatment, suggesting that IPR is not causing significant inhibition of N-demethylation of TMP. In contrast, in a recent study Hegadoren *et al.*(1991) demonstrated that IPR pretreatment resulted in a considerable inhibition of N-deethylation of fenfluramine in rat brain and liver. These results suggest that the length of the N-alkyl side chain as well as the structure of the substrate might have an effect on the ability of IPR to inhibit metabolic N-dealkylation. However, the complete inhibition of the isozyme responsible for alicyclic oxidation (10-oxo) of TMP was the most significant observation from this study.

The present study suggests that at least three different isozymes of cytochrome P450 family are involved in the 2-hydroxylation of TMP and *nor*TMP and 10-oxidation of TMP. Further studies are necessary to establish the identity of these individual isozymes.

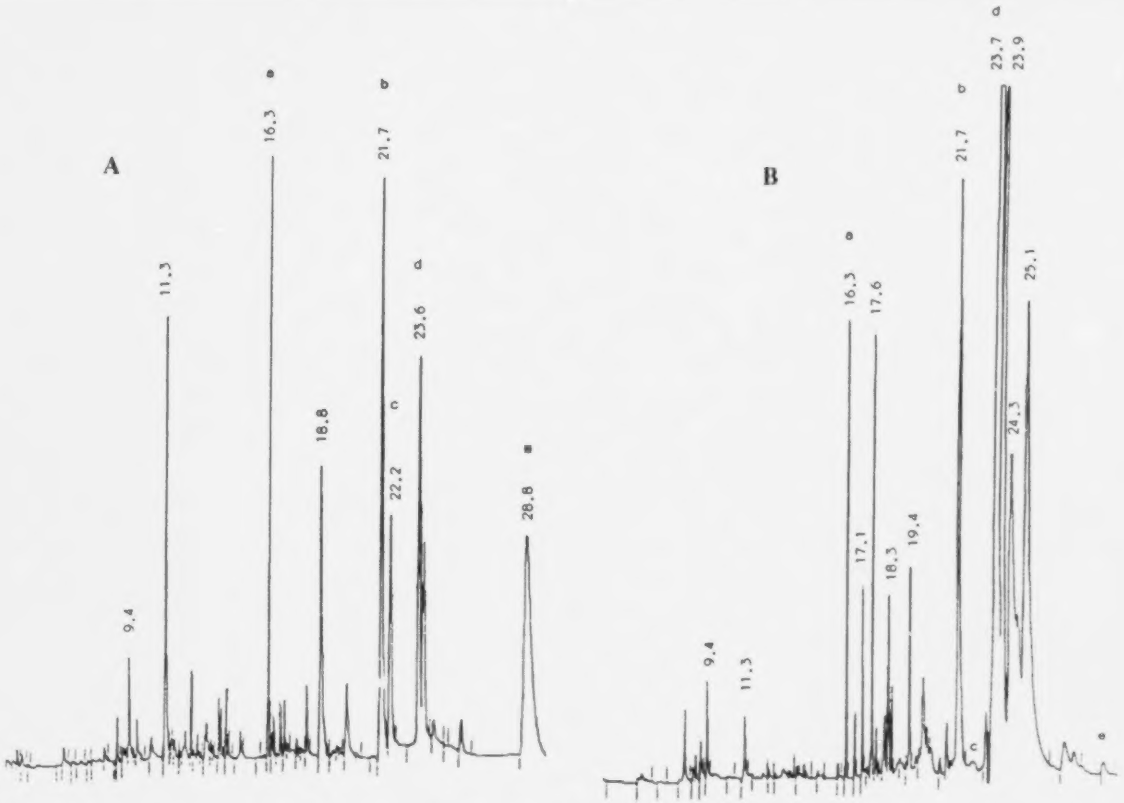


Figure 4-15. Typical GC traces:

A= gas chromatogram of an urine extract from rat treated with TMP alone.
B= gas chromatogram of an urine extract from rat treated with IPR and TMP.

a) TMP **b**) 2-OH-TMP **c**) 10-oxo-TMP
d) 2-OH-norTMP **e**) 10-oxo-2-OH-TMP

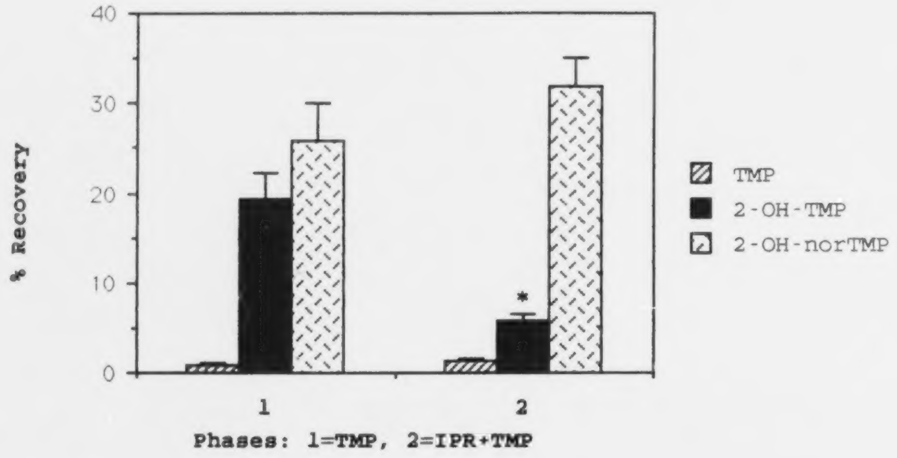


Figure 4-16. Recoveries of TMP and metabolites from rat urine (72h) [Mean ± S.E.M.].

*Significantly different from phase 1 values. $p<0.05$.

4.1.3.2 Effects of QND on the metabolism of TMP

The cytochrome P450 isozyme P450IID6 catalyzes the ring hydroxylation of many aromatic basic drugs of diverse structures (Brosen, 1990; Robbins and Wedlund, 1990; Brosen and Gram, 1989a; Jacqz *et al.*, 1986; Eichelbaum, 1982). The antiarrhythmic drug QND is a potent competitive inhibitor of P450IID6 isozyme (Ayesh *et al.*, 1991; Boobis et al., 1990; Brosen and Gram, 1989a and 1989b; Kobayashi et al., 1989). Therefore, in humans QND strongly inhibits the aromatic ring hydroxylation of NT (Ayesh et al., 1991), IMI (Brosen and Gram, 1989b), DMI (Ayesh et al., 1991; Brosen and Gram, 1989b; Spina et al., 1989), and propranolol (Zhou et al., 1990). In rat QND also inhibits the ring oxidation of methoxyphenamine (Muralidharan et al., 1989b) and amphetamine (Moody et al., 1990). Examples of other drugs whose ring hydroxylation is impaired by QND are listed in section 1.4. QND is a much more potent competitive inhibitor of P450IID6 activity in humans than in rats (Kobayeshi et al., 1989). QND binds strongly to P450IID6 isozyme, but is not itself metabolized by it. QND is metabolized by cytochrome P450IIIA4 isozyme (Guengerich *et al.*, 1986).

The effect of QND on the metabolism of IMI and DMI has only recently been investigated (Ayesh et al., 1991; Brosen and Gram, 1989b). The studies were conducted on healthy human volunteers, and it was found that a dose of 200 mg/day of QND greatly reduced the 2-hydroxylation of IMI and DMI to such an extent that an EM becomes a PM. Similarly, Ayesh *et al.*(1991) have shown that concomitant administration of QND reduces the 2-hydroxylation of both NT and DMI and converts an EM to a PM phenotype. The conclusion from these observations was that ring hydroxylation of IMI, DMI, NT was under the influence of cytochrome P450IID6 isozyme. All studies so far reported on the effect of QND on P450IID6-catalyzed drug oxidative metabolism indicate that it is an effective inhibitor of this metabolic process (Ayesh et al., 1991; Boobis et al., 1990).

This observed inhibitory effect of QND on the 2-hydroxylation of IMI, DMI and NT prompted the investigator to question whether QND would have any effect on the 2-hydroxylation of structurally related drug TMP. Brosen and Gram (1989) have demonstrated that QND inhibits the 2-hydroxylation of IMI and DMI but not the demethylation of IMI. Since TMP undergoes both metabolic 2-hydroxylation and N-demethylation it was of interest to determine what effect QND would have on the metabolism of TMP. So, a study was designed to determine the effect of QND on the overall metabolism of TMP in the rat. The present study would help determine the role of P450IID6 isozyme in the metabolism of TMP.

Urine samples from drug-treated rats were hydrolyzed enzymatically and TMP and its metabolites were extracted from an aliquot of urine samples as described in sections 3.10.4.1

and 3.10.4.3 respectively. Quantitation of TMP metabolites was achieved using a GC-NPD.

Chromatographic separation of TMP, norTMP, 2-OH-TMP, 2-OH-norTMP and the internal standard, IPR, was achieved on a DB-5 fused silica capillary column and the analytical method used was identical to that used in section 4.1.3.1. The extracts from control urines contained no GC peaks that corresponded to those of TMP, its metabolites and the internal standard. Analysis of day-10 urine samples revealed the absence of either TMP or any of its metabolites.

Quantitation of TMP and the three metabolites in 72h urines collected from rats that had received only TMP showed that 2.20 ± 0.52% of the dose was recovered as unchanged TMP, 0.29 ± 0.03% as norTMP, 8.9 ± 0.89% as 2-OH-TMP, and 13.96 ± 1.39% was isolated as 2-OH-norTMP. Pretreatment of rats with a single dose of QND daily for 3 days prior to the administration of TMP altered recoveries of TMP, norTMP, 2-OH-TMP and 2-OH-norTMP to 1.35 ± 0.16%, 0.40 ± 0.09%, 3.27 ± 0.49% and 38.87 ± 2.64%, respectively.

The urinary recoveries of TMP, 2-OH-TMP and 2-OH-norTMP before and after QND treatment are shown in Figure 4-17.

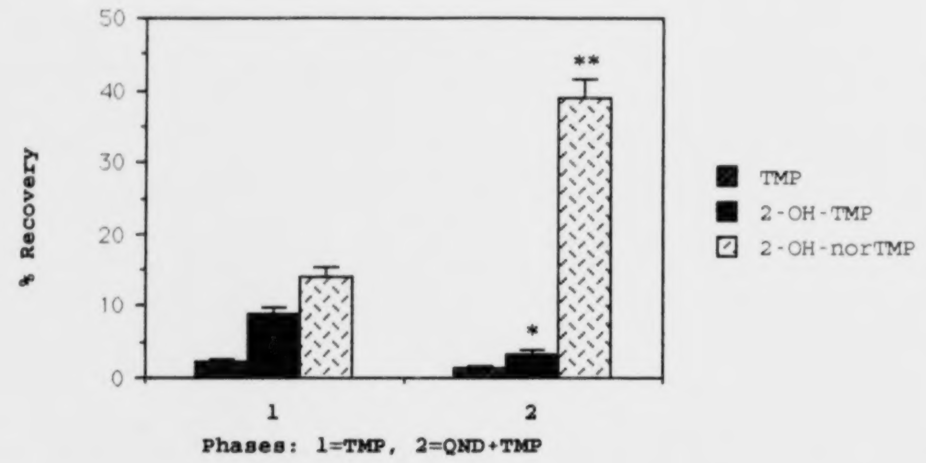


Figure 4-17. Recoveries of TMP and metabolites from rat urine (72h) [Mean ± S.E.M.].

*Significantly different from phase 1 values;

**Significantly different from phase 1 values.

$p<0.05$.

Statistically significant large decreases (relative to vehicle + TMP-treated rats) were observed in the levels of 2-OH-TMP as expected, but, unexpectedly, the 2-OH-norTMP levels were profoundly increased. Clearly the formation of 2-OH-TMP and 2-OH-norTMP are under the influence of two different isozyme systems. As the formation of 2-OH-TMP was significantly reduced by QND pretreatment, it can be concluded that, like IMI, DMI and NT, the 2-hydroxylation of TMP is catalyzed by the cytochrome P450IID6 isozyme. But, since the formation of 2-OH-nor TMP was greatly increased by QND pretreatment, another conclusion from this study is that, the P450IID6 isozyme is **not** involved in the formation of 2-OH-norTMP.

4.1.3.3 Effects of QNN on the metabolism of TMP

Like QND,its diastereoisomer QNN is also an inhibitor of the cytochrome P450IID6 isozyme. It has been demonstrated that QNN is a much less potent inhibitor of cytochrome P450IID6 isozyme in humans (Ayesh et al., 1991; Kobayashi et al., 1989, Muralidharan et al., 1989a). However, in rat QNN is a more potent inhibitor of P450IID6 than is QND (Boobis et al., 1990; Kobayashi et al., 1989). This is because the active site of debrisoquine 4-hydroxylase, i.e. P450IID6, in the rat is qualitatively different from that of the human isozyme (Boobis et al., 1986).

It was of interest to see the effects of QNN on the *in vivo* metabolism of TMP in the rat. The study was conducted according to the protocol described in section 3.1.3.2 for QND, except that in place of QND, a po dose of QNN was administered. The dose of TMP remained same. Similar extraction, derivatization and assay methods were employed as stated in section 3.1.3.2.

The bar graph illustrating the % recovery of TMP and two metabolites in 72h urine is supplied (Figure 4-18). Quantitation of TMP and metabolites in urine extracts from rats that had received only TMP showed that 2.06 ± 0.38% was recovered as unchanged TMP, 0.49 ± 0.08% as norTMP, 7.66 ± 0.83% as 2-OH-TMP and 16.39 ± 1.16% was isolated as 2-OH-norTMP. Pretreatment of rats with three doses of QNN altered the % recovery of the metabolites. Therefore, after QNN pretreatment, 1.75 ± 0.29% was recovered as unchanged TMP, 0.27 ± 0.05% as norTMP, 1.85 ± 0.31% as 2-OH-TMP and 28.33 ± 3.03% as 2-OHnorTMP.

Pretreatment of rats with QNN resulted in a greater than 400% decrease in the 72h-urinary excretion of 2-OH-TMP, while pretreatment of rats with QND (section 4.1.3.2) resulted in a 271% decrease in the formation of 2-OH-TMP. These results clearly demonstrate that in rat QNN is a more potent inhibitor of P450IID6 isozyme than is QND. This is in agreement with other published results (Boobis et al., 1990; Kobayashi et al., 1989).

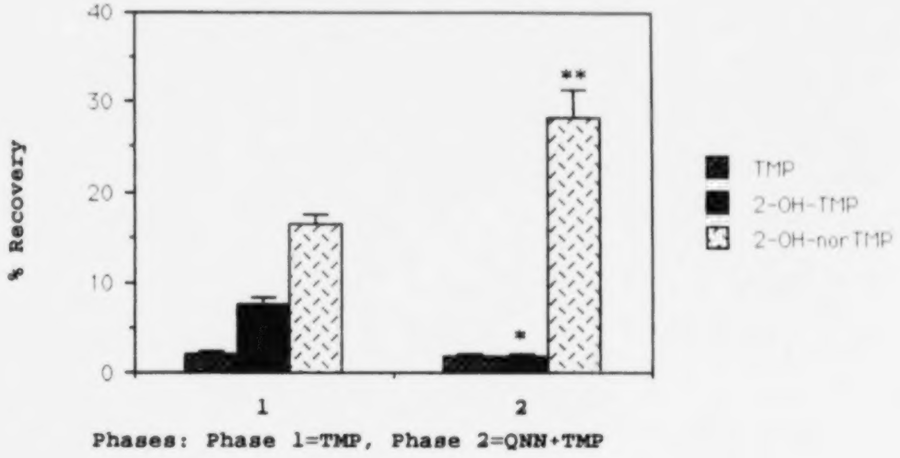


Figure 4-18. Urinary excretion (72h) of TMP and metabolites in rat [Mean ± S.E.M.].

*Significantly different from phase 1 values.

**Significantly different from phase 1 values.

$p<0.05$.

Like QND, QNN pretreatment also resulted in a significant decrease in the formation of 2-OH-TMP, suggesting again that P450IID6 isozyme is involved in the aromatic hydroxylation of TMP. Since QNN pretreatment also resulted in an increase in the formation of 2-OH-norTMP, it can be concluded that metabolic formation of 2-OH-norTMP is **not** under the influence of P450IID6 isozyme.

4.2 Studies conducted on 2-OH-TMP

4.2.1 Urinary metabolites of 2-OH-TMP in the rat

This study, as well as the study described latter in section 4.3, were performed in order to delineate the *in vivo* pathway of formation of 2-OH-norTMP in the rat. It has been demonstrated in sections 4.1.3.1 and 4.1.3.2 that challenging rats with QND and QNN prior to TMP administration increased the metabolic formation of 2-OH-norTMP; at the same time the formation of 2-OH-TMP was markedly reduced. These observations suggested that different isozymes of the cytochrome P450 family are involved in the formation of 2-OH-TMP and 2-OH-norTMP. It is probable that 2-OH-norTMP is formed metabolically either from 2-OH-TMP by demethylation, or from norTMP by aromatic hydroxylation. It was of interest to investigate the pathway of formation of 2-OH-norTMP by dosing rats separately with 2-OH-TMP and norTMP. Studies conducted on 2-OH-TMP are described here, while those with norTMP are described in the following section 4.3.

Rats were dosed ip with 2-OH-TMP fumerate and urine samples were collected as described in section 3.11.1. The urine samples were enzymatically hydrolyzed and extracted as stated in section 3.11.2. The metabolites were identified by interpretation of the fragmentation pattern apparent in each mass spectrum and also by comparison of mass spectra with those of authentic metabolites. The identity of each metabolite was also confirmed by comparing the retention time of each *in vivo* metabolite with that of the authentic sample.

The identified major *in vivo* metabolite of 2-OH-TMP was 10-*oxo*-2-OH-TMP. Another metabolite was 2-OH-norTMP, but it was isolated in low amounts. Quantitation of 2-OH-TMP and 2-OH-norTMP was performed using a GC-NPD. TMP was used as the internal standard.

The results of the quantitative study showed that 30.78 ± 4.32% of the dose was recovered as unchanged 2-OH-TMP and only 4.39 ± 0.56% was isolated as 2-OH-norTMP. Due to the nonavailability of an authentic sample of 10-*oxo*-2-OH-TMP, the quantitation of this metabolite was not performed.

These quantitative results show that the substrate 2-OH-TMP was excreted mainly in the unchanged form. The amount of 2-OH-norTMP formed was very little. It is apparent from the above study that demethylation of 2-OH-TMP to 2-OH-norTMP is not a dominant metabolic pathway in the rat. An alternate pathway for the metabolic formation of 2-OH-norTMP might be the aromatic hydroxylation of norTMP. With a view to exploring the validity of this hypothesis the following study was conducted.

4.3 Studies conducted on norTMP

4.3.1 *In vivo* metabolic study in rat

The major *in vivo* metabolites of norTMP isolated were 2-OH-norTMP and 10-OH-norTMP together with a small amount of unchanged norTMP. Essentially most of the administered norTMP was metabolized by aromatic hydroxylation to 2-OH-norTMP and alicyclic oxidation to 10-OH-norTMP. The result of the quantitative study showed that 0.94 ± 0.06% of the dose was recovered as unchanged norTMP, 21.87 ± 2.12% as 2-OH-norTMP and probably a similar amount was excreted as 10-OH-norTMP. Because of the non-availability of authentic 10-OH-TMP sample, quantitation of only norTMP and 2-OH-norTMP was performed. However, the peak areas of 10-OH-TMP and 2-OH-norTMP in the GC trace were similar, suggesting that approximately equal amounts of each metabolite were formed *in vivo*.

It is clear from the above study that the major metabolic pathway for the formation of 2-OH-norTMP in rat is aromatic hydroxylation of norTMP and not the demethylation of 2-OH-TMP. This is in agreement with the results obtained from the *in vitro* studies described in section 4.1.2.1. It is concluded that the major precursor of 2-OH-norTMP is norTMP.

It was demonstrated in section 4.1.3.2 that pretreatment of rats with QND greatly increased the aromatic hydroxylation of norTMP. It was pertinent at this stage to investigate the effect of QND pretreatment on the metabolism of norTMP in the rat.

4.3.2 Effects of QND on the metabolism of norTMP

The study was conducted in three phases with intermittent washout periods as described in section 3.11.1.

The urine samples from drug-treated rats were hydrolyzed and norTMP and metabolites were extracted as described in section 3.11.2. Quantitation of norTMP and the metabolite 2-OH-norTMP was performed utilizing a GC-NPD. The assay method used is described in section 3.11.3. No interfering peaks were observed in the analytical region of the GC chromatogram of the urine extracts from drug-naive rats or from QND-treated rats.

Quantitation of norTMP and its metabolite 2-OH-norTMP in 72h urines collected from rats that had received only norTMP showed that 0.94 ± 0.0.06% of the dose was recovered as unchanged norTMP, and 21.87 ± 2.12% was isolated as 2-OH-norTMP. Pretreatment of rats with three doses of QND prior to

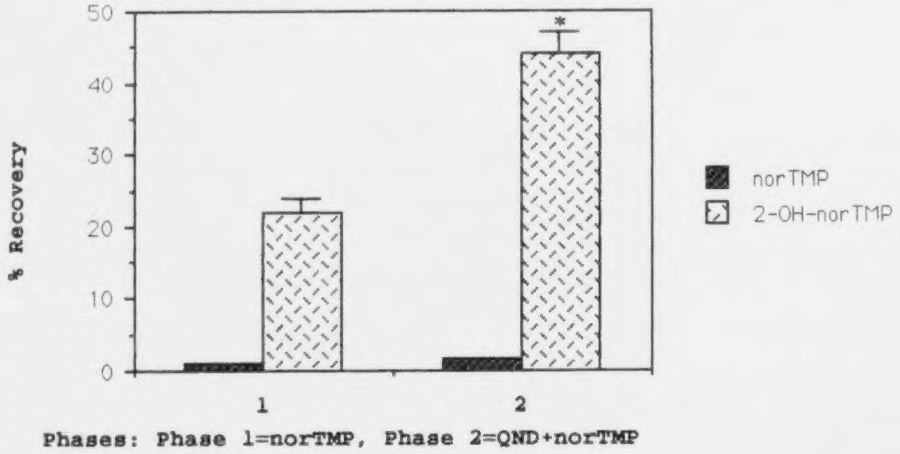


Figure 4-19. Recoveries of norTMP and its metabolite from rat urine (72h) [Mean ± S.E.M.].
*Significantly different from phase 1 values. $p<0.05$.

norTMP administration altered the recoveries of norTMP and 2-OH-norTMP to 1.48 ± 0.15% and 44.12 ± 3.0% respectively. Therefore, QND pretreatment resulted in a marked increase in the metabolic formation of 2-OH-norTMP. These results agree with those obtained in section 4.1.3.1.

QND is a known inhibitor of cytochrome P450IID6-catalyzed drug oxidations, including aromatic oxidation of phenolic metabolites (Boobis et al., 1990). Both QND and QNN significantly inhibited the formation of 2-OH-TMP, but at the same time the formation of 2-OH-norTMP was increased (sections 4.1.3.1 and 4.1.3.2). In contrast, aromatic hydroxylation of IMI and DMI are under the influence of the same cytochrome P450IID6 isozyme and both metabolic pathways are inhibited by prior administration of QND (Ayesh et al., 1991; Brosen and Gram, 1989b). Since the aromatic hydroxylation of norTMP is significantly enhanced in the presence of QND, it can be concluded that unlike DMI, the aromatic hydroxylation of norTMP to a phenolic metabolite is under the influence of an isozyme other than cytochrome P450IID6.

4.4 Studies conducted on IPR

4.4.1 In vivo metabolism study

The underivatized and the acetylated metabolites of IPR were separated using gas chromatography and their chemical structures were deduced by detailed interpretations of the fragmentation patterns apparent in each EIMS.

No authentic reference samples of any of IPR's metabolites were available. Thus, to assist in the identification of the urinary metabolites of this drug in the rat, an efficient isolation/gas chromatographic separation procedure was developed for IPR metabolites, during which the mass spectra of each metabolite and its acetylated derivative were recorded and the chemical structures of these metabolites were deduced from their fragmentation pathways.

4.4.1.1 Mass spectral observations of general application

Information on the mass spectral behavior of IPR and some of its simple derivatives is required at the outset to permit comparative interpretations of spectra of metabolites. The EI mass spectrum of the parent drug IPR contains eight diagnostic ions of *m/z* 284 $[M^{+\cdot}]$, 213, 212, 171, 170, 145, 144 and 58 (base peak), and appropriate structures for these ions are suggested in Figure 4-20. Of particular diagnostic value is the presence of ions of *m/z* 213 $[M\text{-}71]^+$, 212 $[M\text{-}72]^+$ and 58. The first two ions are indicative of an intact ring system, while the ion of *m/z* 58 is derived from the 5-[3-dimethylaminopropyl] side-chain of IPR. The presence of an abundant fragment ion of *m/z* 58 in the mass spectrum of an IPR metabolite will indicate that the metabolite retains a terminal $CH_2N(CH_3)_2$ moiety on the side-chain attached to the ring N atom. By employing similar reasoning, the spectrum of norIPR, the product of metabolic mono-N-demethylation of IPR which has a $CH_2CH_2CH_2NHCH_3$ chain attached at the ring N atom, would be expected to contain diagnostic $[M-57]^+$ and $[M-58]^+$ fragment ions, but no *m/z* 58 fragment, and this is what occurs (Peak 517N; Table 4-1). The mass spectra of other IPR metabolites that possess a $CH_2CH_2CH_2NHCH_3$ side-chain at N_5, therefore, should be readily identifiable because of the presence of $[M-57]^+$ and $[M-58]^+$ fragments in their EI mass spectra.

The fragment ions of *m/z* 171, 170, 145, and 144 in the EIMS of IPR are also of diagnostic value. The mass spectra of all metabolites which possess an underivatized aromatic ring should provide EIMS which contain these fragment ions.

Acetylation of drug metabolites prior to combined GC-MS analysis can be a very important procedure in the identification of a metabolite's structure. If acetylation is performed under anhydrous conditions, primary and secondary NH groups and alcoholic and phenolic OH groups are all acetylated. Of particular relevance to the present study are drugs and metabolites that possess a $CH_2CH_2CH_2NHCH_3$ side-chain. On acetylation, this side-chain is converted to a $CH_2CH_2CH_2N(CH_3)COCH_3$ moiety, and a drug or metabolite that possesses this grouping will produce an EI mass spectrum in which a fragment ion of *m/z* 114 is prominent and often the base peak. Such is the case with N-acetylated-DMI

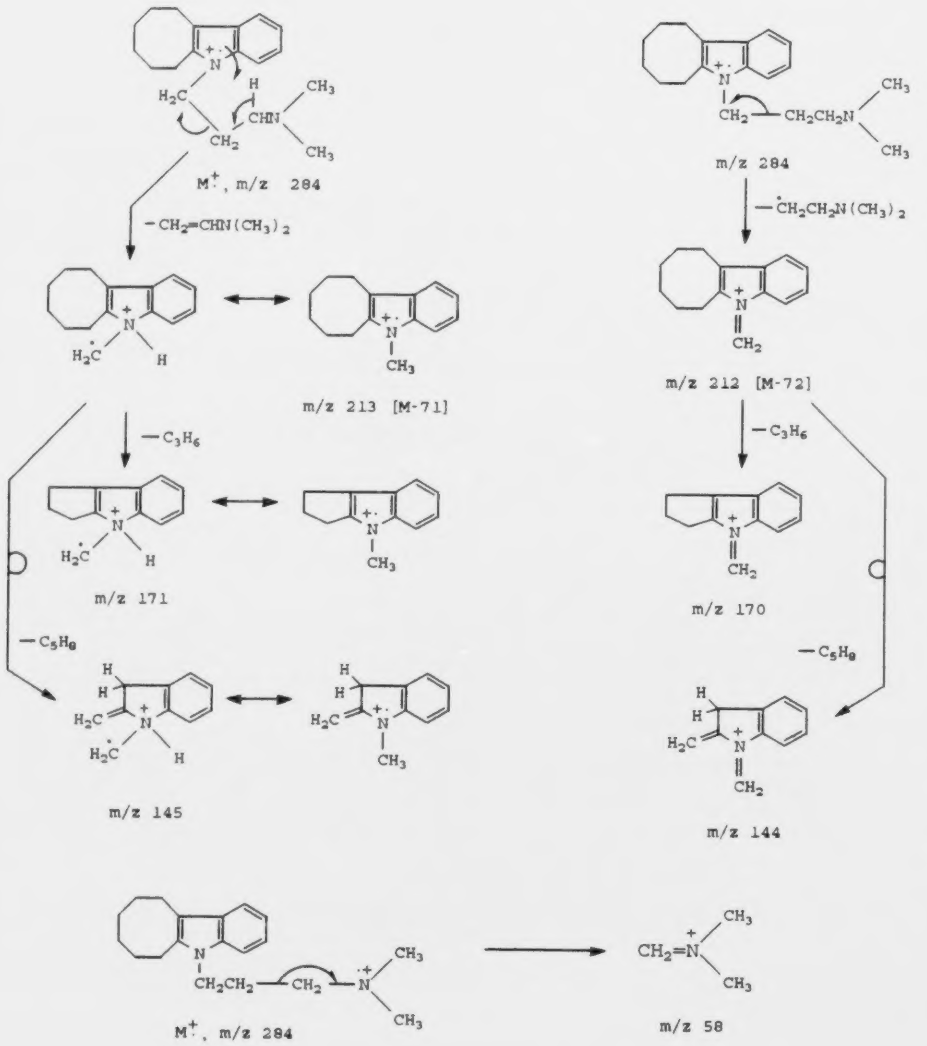

M+·, m/z 284
− CH2=CHN(CH3)2
m/z 213 [M-71]
− C3H6
m/z 171
− C5H8
m/z 145
m/z 284
− ·CH2CH2N(CH3)2
m/z 212 [M-72]
− C3H6
m/z 170
− C5H8
m/z 144
M+·, m/z 284
m/z 58

Figure 4-20. Mass fragmentation pathways of iprindole (IPR).

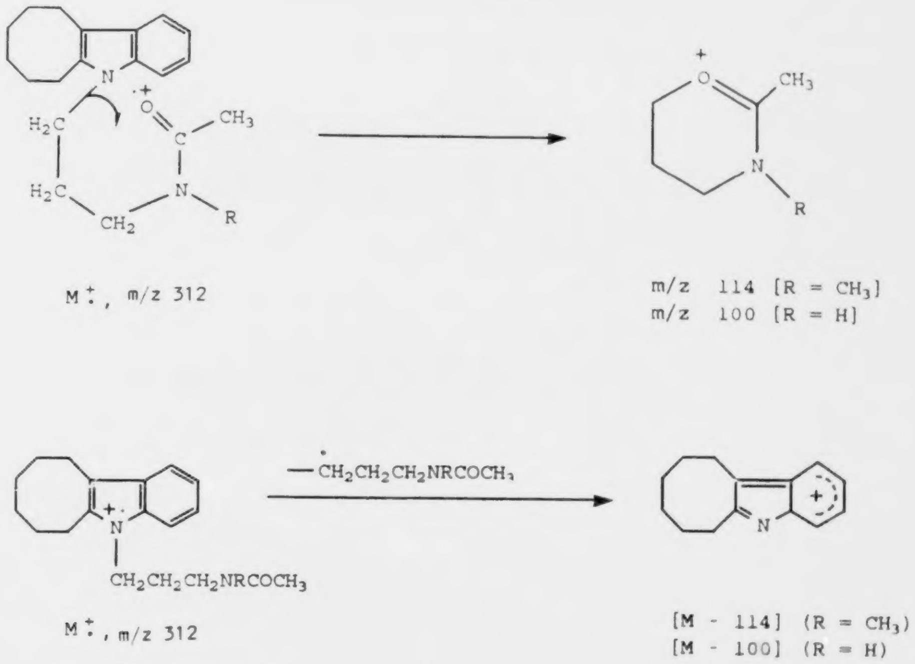

N
·+
O
H2C
C
CH3
H2C
N
R
CH2
M+·, m/z 312
+
O
CH3
N
R
m/z 114 [R = CH3]
m/z 100 [R = H]
+·
N
CH2CH2CH2NRCOCH3
M+·, m/z 312
— ·CH2CH2CH2NRCOCH3
+
N
[M - 114] (R = CH3)
[M - 100] (R = H)

Figure 4-21. Fragmentation pathways of N-demethylated and N,N-didemethylated IPR acetate.

(Carrington & Frigerio, 1977; Coutts & Jones, 1980). The mechanism of formation of the *m/z* 114 fragment is illustrated in Figure 4-21. In addition to the *m/z* 114 ion, the complementary $[M-114]^+$ fragment and an $[M-100]^+$ ion $[M-CH_2CH_2N(CH_3)COCH_3]^+$ will also be present in the mass spectra of acetylated metabolites that possess a $CH_2CH_2CH_2N(CH_3)COCH_3$ side-chain. Both ions are prominent, for example, in the spectrum of N-acetyldesipramine (Carrington and Frigerio, 1977). The $[M-100]^+$ ion in the spectrum of N-acetyl-norIPR (#953A) is of mass *m/z* 212, and is identical to the ion of that mass in Figure 4-20.

In a similar manner, acetylated drugs and metabolites that contain a $CH_2CH_2CH_2NHCOCH_3$ group on the heterocyclic N atom may be expected to produce EI mass spectra that contain an abundant ion of *m/z* 100 and the complementary $[M-100]^+$ fragment ion (Figure 4-21). In contrast, the equivalent ions of *m/z* 128 and $[M-128]^+$ in the spectrum of acetylated N-demethylated TMP which has a branched $CH_2CH(CH_3)CH_2NHCOCH_3$ group located on the heterocyclic N atom are of low abundance and of little diagnostic value (EIMS #3, Figure 4-3, section 4.1.1.1). Branching in the side-chain is apparently a deterrent to this fragmentation pathway.

Phenolic and alcoholic O-acetates can also be distinguished by their EI mass spectral behavior. Phenolic acetates readily expel a ketene molecule (42 amu) in the manner indicated in Figure 4-22a.

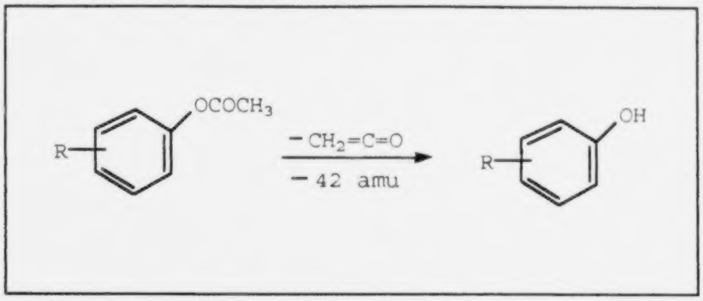


Figure 4-22a. Expulsion of a ketene molecule from a cyclic phenolic acetate.

Acetic acid (60 amu) is not eliminated during fragmentation of phenolic acetates. In contrast, it is known that 1,2-elimination of acetic acid is a dominant process in acetates of cyclic alcohols (Budzikiewicz *et al.*, 1967) in the manner shown in Figure 4-22b. Such esters do not fragment by expelling ketene.

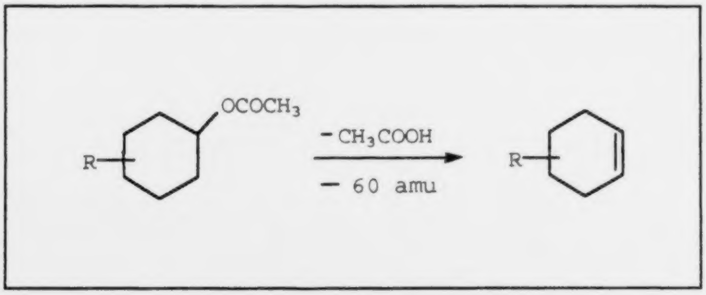


Figure 4-22b. Expulsion of an acetic acid molecule from a cyclic alcoholic acetate.

4.4.1.2 Identification of site of metabolic aliphatic (or alicyclic) oxidation

The GC-MS behavior of acetates formed by anhydrous acetylation of metabolically produced alcohols is of value in identifying the site of aliphatic (or alicyclic) oxidation. Acetylated aliphatic and alicyclic alcohols readily expel a molecule of acetic acid, corresponding to dehydration of the original alcohol, only if a conjugated product results. This dehydration is most likely in allylic systems. In contrast, vinylic acetates are much more resistant to the expulsion of acetic acid during GC analysis. Some examples are listed below.

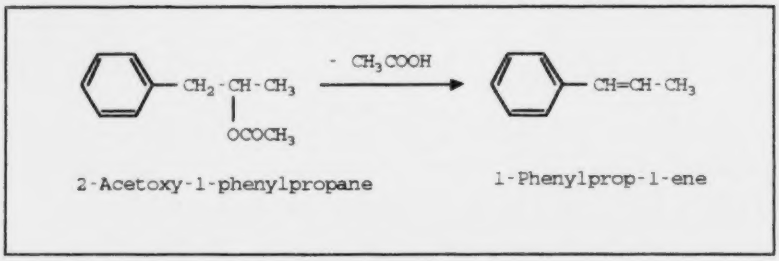
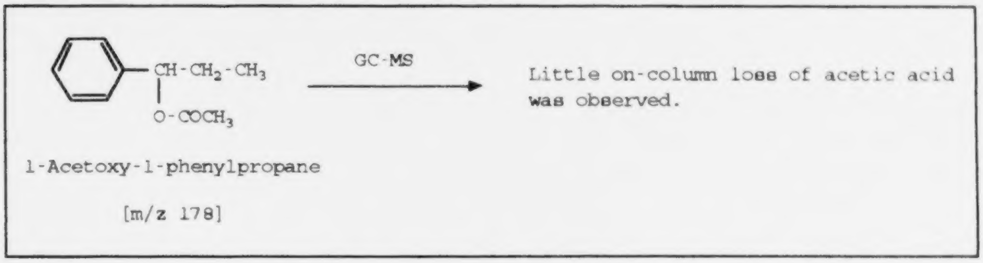


Figure 4-23a. Loss of acetic acid molecule from 2-acetoxy-1-phenylpropane.

1. Anhydrous acetylation (section 3.9.2) of 2-hydroxy-1-phenylpropane produced 2-acetoxy-1-phenylpropane. When the product, 2-acetoxy-1-phenylpropane, was chromatographed under conditions identical to those used for the separation of IPR metabolites (section 3.12.5), the on-column expulsion of

acetic acid was complete and the product detected by the mass spectrometer was 1-phenylprop-1-ene (Figure 4-23a).

In contrast, when the isomer 1-acetoxy-1-phenylpropane, a vinylic acetate, was subjected to GC, it was detected mainly intact; very little on-column loss of acetic acid was observed (Figure 4-23b).


CH-CH$_2$-CH$_3$
O-COCH$_3$
GC-MS
1-Acetoxy-1-phenylpropane
[m/z 178]

Little on-column loss of acetic acid was observed.

Figure 4-23b. GC-MS behavior of 1-acetoxy-1-phenylpropane.

2. When cholesterol acetate was similarly subjected to GC-MS analysis, complete loss of acetic acid was observed, with the formation of a conjugated product (Figure 4-23c).

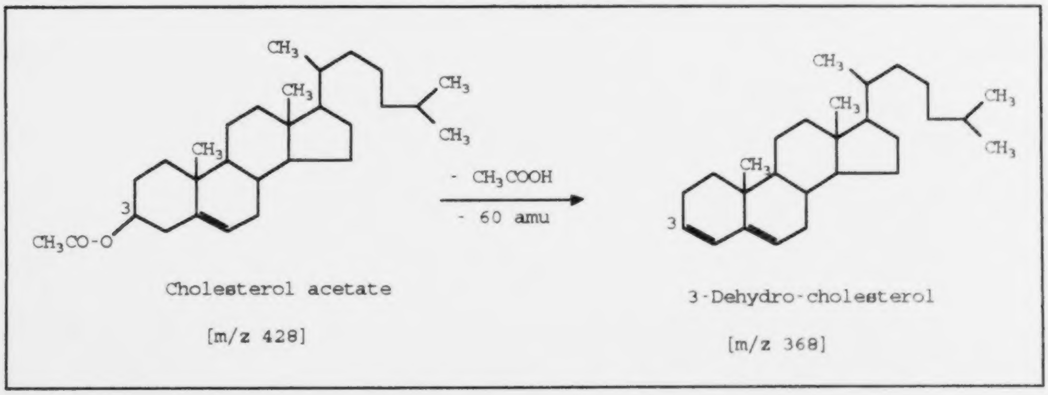

CH$_3$
CH$_3$
CH$_3$
CH$_3$
CH$_3$
3
CH$_3$CO-O
- CH$_3$COOH
- 60 amu
Cholesterol acetate
[m/z 428]
CH$_3$
CH$_3$
CH$_3$
CH$_3$
CH$_3$
3
3-Dehydro-cholesterol
[m/z 368]

Figure 4-23c. Loss of acetic acid molecule from cholesterol acetate.

Similarly, β-sitosterol acetate lost a molecule of acetic acid (Figure 4-23d) when subjected to GC-MS, using conditions identical to those used for the separation of IPR metabolites.

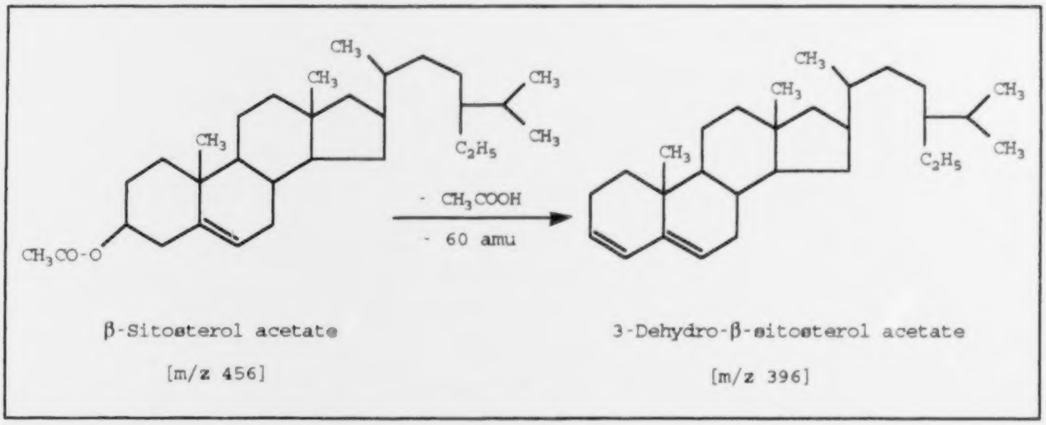

CH3
CH3
CH3
CH3
CH3
C2H5
CH3CO-O
- CH3COOH
- 60 amu
CH3
CH3
CH3
CH3
CH3
C2H5
β-Sitosterol acetate
[m/z 456]
3-Dehydro-β-sitosterol acetate
[m/z 396]

Figure 4-23d. Loss of acetic acid molecule from β-sitosterol acetate.

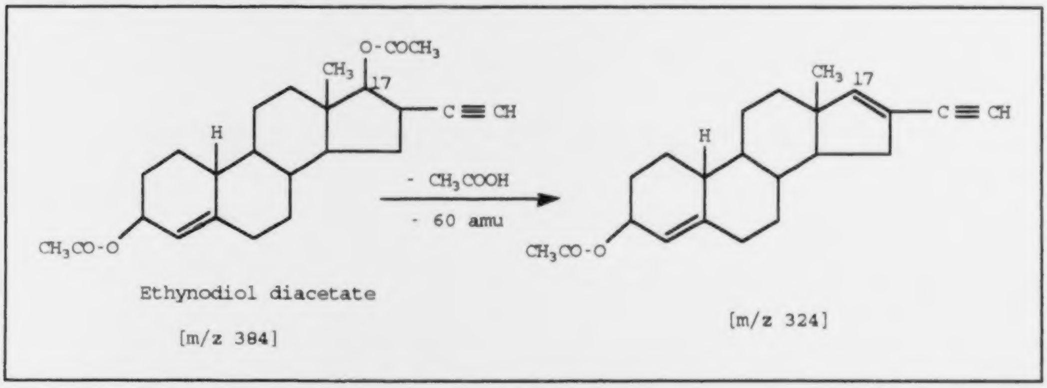

O-COCH3
CH3
17
C ≡ CH
H
CH3CO-O
- CH3COOH
- 60 amu
CH3 17
C ≡ CH
H
CH3CO-O
Ethynodiol diacetate
[m/z 384]
[m/z 324]

Figure 4-23e. GC-MS behavior of ethynodiol diacetate.

3. However, when ethynodiol diacetate was chromatographed under conditions identical to those used for the separation of IPR metabolites, its mass spectrum possessed a molecular ion and the expulsion of one of the acetate groups as acetic acid was not complete (Figure 4-23e).

It is even possible that the partially expelled acetate moiety was the one located at the C_{17} position of the ethynodiol diacetate molecule, because this expulsion of acetic acid would result in a conjugated product.

4.4.1.3 GC-MS spectral studies

GC traces of underivatized and acetylated IPR metabolites were recorded under identical GC-MS conditions, and are provided (Figures 4-24 and 4-25). GC peaks of IPR and its metabolites were detected in both traces, but the resolution of peaks was much superior in the trace of acetylated metabolites (Figure 4-25). Information derived from the mass spectra of acetylated metabolites was of more value in the elucidation of the chemical structures of metabolites (see Table 4-1). Confirmatory interpretive information is obtainable, however, from the mass spectra of nonacetylated metabolites.

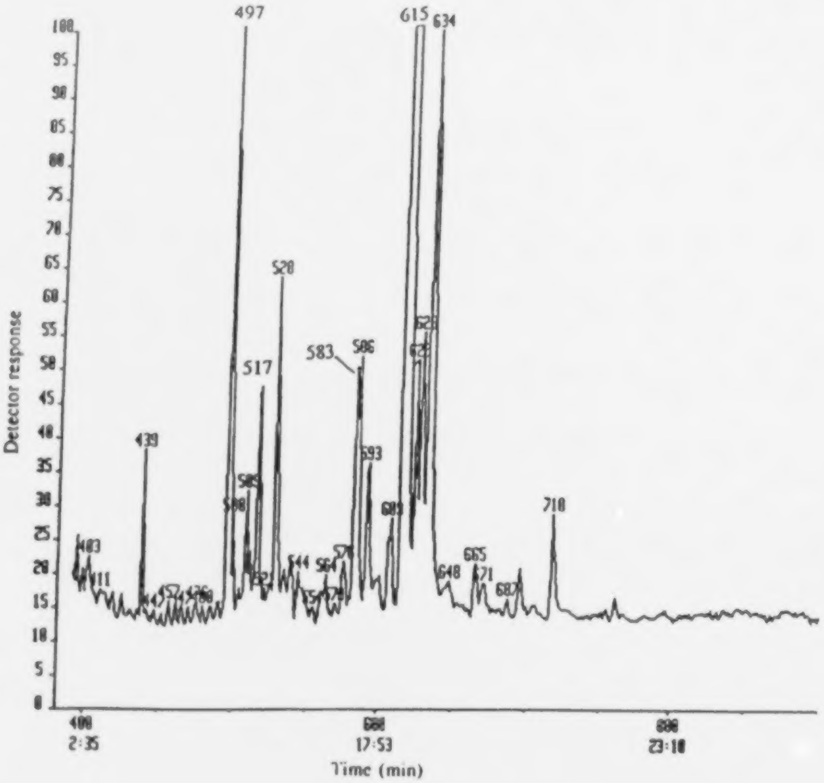


Figure 4-24. Total ion current chromatogram of a non-acetylated urine extract from a rat dosed intraperitoneally with iprindole.

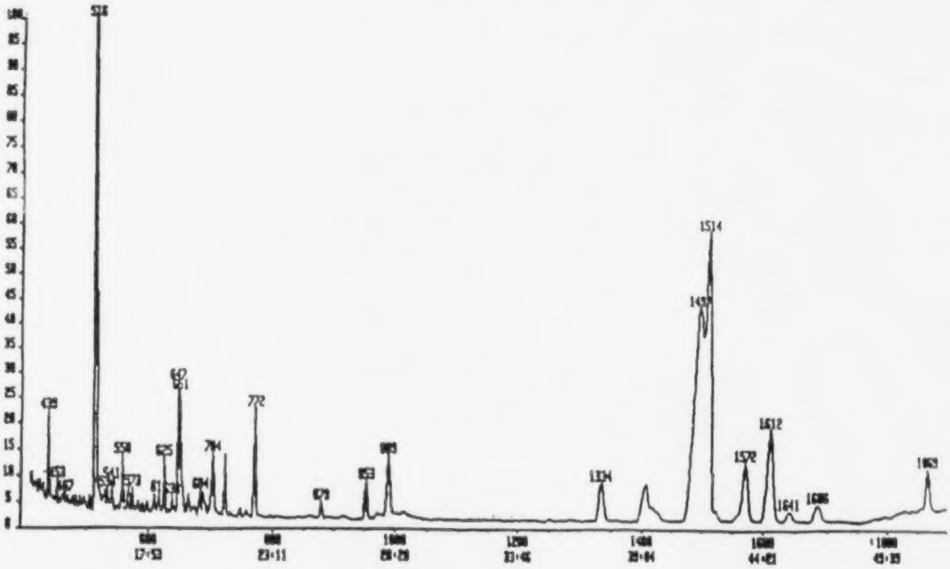


Figure 4-25. Total ion current chromatogram of an acetylated urine extract from a rat dosed intraperitoneally with iprindole.

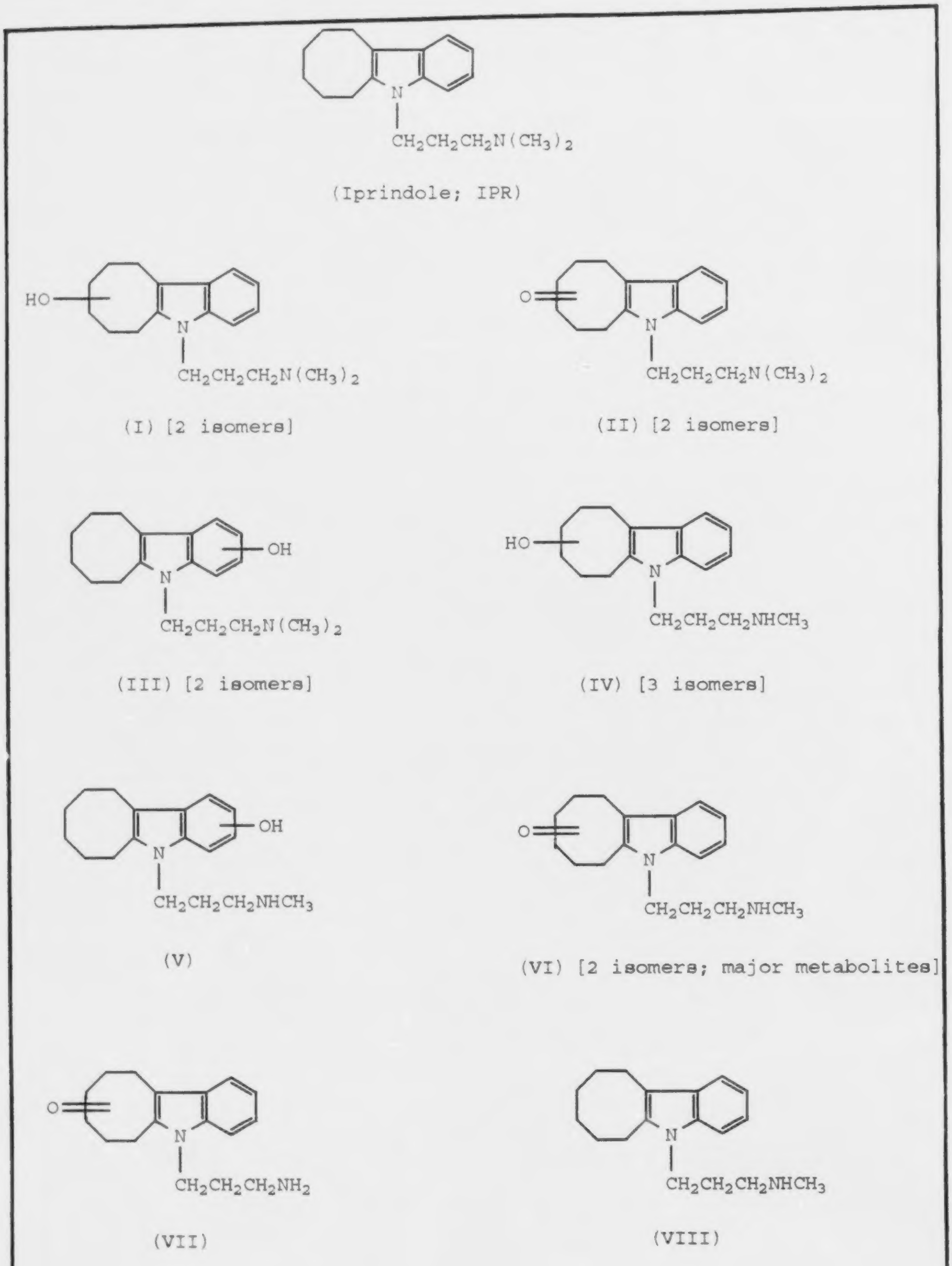
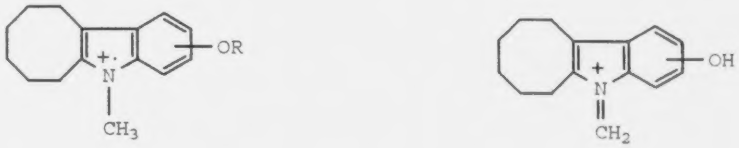

N
CH2CH2CH2N(CH3)2
(Iprindole; IPR)
HO
N
CH2CH2CH2N(CH3)2
(I) [2 isomers]
O
N
CH2CH2CH2N(CH3)2
(II) [2 isomers]
OH
N
CH2CH2CH2N(CH3)2
(III) [2 isomers]
HO
N
CH2CH2CH2NHCH3
(IV) [3 isomers]
OH
N
CH2CH2CH2NHCH3
(V)
O
N
CH2CH2CH2NHCH3
(VI) [2 isomers; major metabolites]
O
N
CH2CH2CH2NH2
(VII)
N
CH2CH2CH2NHCH3
(VIII)

Figure 4-26. Proposed structures of IPR metabolites in rat urine.


OR
+.
N
CH3
OH
+
N
CH2

m/z 271 [M-71], R = $COCH_3$
m/z 229 [M-71], R = H

m/z 228

Figure 4-27. Structures of fragment ions of IPR metabolites.

Table 4-1. Mass spectral data on iprindole metabolites in rat (percentage relative abundances are given in parenthesis).

a) Non-acetylated (N) metabolites (see Figure 4-24):

*Peak # 497N: 284 (35) [M^+, $C_{19}H_{28}N_2$]; 213 (25) [$C_{15}H_{19}N$] ; 212 (14); 171 (11) [$C_{12}H_{13}N$]; 170 (46) [$C_{12}H_{12}N$]; 145 (15) [$C_{10}H_{11}N$]; 144 (12) [$C_{10}H_{10}N$]; 58 (100) [C_3H_8N].

*Peak # 517N: 270 (100) [M^+]; 213 (45); 212 (53); 198 (34); 183 (36) [$C_{13}H_{13}N$]; 182 (26) [$C_{13}H_{12}N$]; 171 (23); 170 (90); 145 (35); 144 (42); 143 (25) [$C_{10}H_9N$].

Peak # 576N: 300 (24) [M^+]; 282 (4); 229 (9); 198 (5); 197 (17); 170 (10); 158 (33); 144 (23); 58 (100).

Peak # 583N: 298 (15) [M^+]; 227 (11); 226 (5); 171 (18); 170 (16); 58 (100).

Peak # 586N: 298 (24) [M^+]; 227 (20); 199 (14); 198 (11); 171 (14); 170 (23); 58 (100).

Peak # 593N: 300 (18) [M^+]; 282 (3); 229 (12); 198 (10); 170 (17); 144 (17); 58 (100).

*Peak # 609N: 284 (37) [M^+]; 227 (32); 226 (10); 199 (12) [$C_{14}H_{17}N$]; 198 (17) [$C_{14}H_{16}N$]; 184 (19) [$C_{13}H_{14}N$]; 144 (33); 143 (100) [$C_{10}H_9N$].

Peak # 629N: 300 (45) [M^+]; 282 (14); 229 (43); 228 (30); 186 (50); 58 (100).

Peak # 615N: 284 (47) $[M^+]$; 227 (33); 226 (14); 199 (12); 198 (11); 184 (23); 171 (67); 170 (100); 156 (23); 155 (26); 144 (16); 143 (17).

Peak # 626N: 286 (63) $[M^+]$; 268 (8); 229 (29); 228 (28); 198 (24); 171 (22); 170 (59); 169 (21); 158 (40); 145 (35); 144 (100).

Peak # 634N: 286 (100) $[M^+]$; 268 (5); 229 (40); 228 (73); 216 (31); 185 (30); 171 (30); 170 (64); 169 (30); 158 (64); 145 (37); 144 (92); 130 (30).

Peak # 665N: 286 (81) $[M^+]$; 229 (65); 228 (80); 198 (43); 186 (100).

b) Acetylated (A) metabolites (see Figure 4-25):

Peak # 516A: 284 (35) $[M^+]$; 213 (31) [M-71]; 212 (11) [M-72]; 171 (9); 170 (37); 145 (13); 144 (9); 58 (100).

Peak # 625A: 282 (35) $[M^+]$; 211 (34) [M-71]; 210 (16) [M-72]; 182 (10); 58 (100).

Peak # 647A: 298 (21) $[M^+]$; 227 (12) [M-71]; 226 (5) [M-72]; 199 (5); 198 (5); 171 (23); 170 (20); 58 (100).

Peak # 651A: 298 (20) $[M^+]$; 227 (14) [M-71]; 226 (5) [M-72]; 199 (13); 198 (10); 171 (15); 170 (20); 58 (100).

Peak # 704A: 342 (24) $[M^+]$; 271 (10) [M-71]; 212 (12); 211 (41); 170 (20); 144 (13); 58 (100).

Peak # 723A: 342 (26) [M$^+$]; 271 (18) [M-71]; 229 (38); 228 (30); 186 (18); 58 (100).

Peak # 772A: 342 (34) [M$^+$]; 271 (26) [M-71]; 229 (38); 228 (48); 186 (16); 58 (100).

Peak # 953A: 312 (70) [M$^+$]; 284 (14); 157 (15); 212 (64); 198 (21) [M-114]; 170 (24); 144 (19); 114 (100).

Peak # 1334A: 370 (20) [M$^+$]; 310 (58); 270 (6); 210 (42); 196 (24); 168 (12); 144 (11); 114 (100).

Peak # 1408A: 312 (100) [M$^+$]; 284 (11); 255 (54); 226 (27); 212 (9) [M-100]; 170 (33); 157 (38); 156 (37); 143 (15); 142 (20); 100 (97).

*Peak # 1497A: 326 (68) [M$^+$, $C_{20}H_{26}N_2O_2$]; 298 (8) [$C_{18}H_{22}N_2O_2$]; 269 (24) [$C_{17}H_{21}N_2O$]; 226 (20) [$C_{15}H_{16}NO$]; 212 (7) [M-114, $C_{14}H_{14}NO$]; 170 (22) [$C_{12}H_{12}N$]; 114 (100) [$C_6H_{12}NO$].

*Peak # 1514A: 326 (67) [M$^+$, $C_{20}H_{26}N_2O_2$]; 298 (9) [$C_{18}H_{22}N_2O_2$]; 226 (30) [$C_{15}H_{16}NO$]; 212 (8) [M-114, $C_{14}H_{14}NO$]; 198 (18) [$C_{14}H_{16}N$]; 184 (24) [$C_{13}H_{14}N$]; 170 (26) [$C_{12}H_{12}N$]; 114 (100) [$C_6H_{12}NO$].

Peak # 1572A: 370 (42) [M$^+$]; 310 (14); 270 (17); 210 (17); 196 (20); 144 (14); 114 (100).

Peak # 1612A: 370 (40) [M$^+$]; 310 (6); 282 (14); 270 (17); 210 (10); 196 (9); 170 (11); 144 (10); 114 (100).

Peak # 1869A: 370 (43) [M^+]; 328 (14); 315 (22); 296 (11); 270 (12); 253 (24); 228 (33); 196 (12); 114 (100).

* Accurate mass measurements were determined to confirm the elemental compositions of fragment ions in the spectra of IPR (# 497N) and its most abundant metabolites (acetylated derivatives # 1497A and 1514A), and of selected ions in the spectra of metabolites # 517N and 609N.

4.4.1.4 Structure elucidation

Peak # 516A (Figure 4-25). This peak had GC and mass spectral properties identical to those of peak #497N (Figure 4-24), indicating that this metabolite does not acetylate. The mass spectrum of an authentic sample of IPR was also identical. This peak is identified as IPR.

Peak # 625A (Figure 4-25). The mass spectrum of this peak was very simple and contained only five ions of relative abundance 10% or greater: *m/z* 282 [$M^{+\cdot}$], 211 $[M-71]^+$, 210 $[M-72]^+$, 182 and 58. The presence of $[M-71]^+$, $[M-72]^+$ and *m/z* 58 ions indicates the retention of an intact 5-[3-dimethylaminopropyl] side-chain. The mass of this metabolite is 2 amu less than that of IPR. It is clearly a dehydrogenated derivative of IPR. The resulting double bond must be located in the cyclooctyl ring, but its location is uncertain. Based on the experience with the metabolism of TMP (section 4.1.1.1), the IPR metabolite 625A is not a primary metabolite. The initial metabolite is most likely to be an alicyclic ring monohydroxylated species that is readily dehydrated after acetylation and during its subsequent GC analysis. As ready dehydration only occurs if a conjugated product results (Section 4.4.1.2), the double bond in the alicyclic ring must be located in the 6-7 or 10-11 positions. Thus the primary metabolite is identified as 6-, *or* 7- *or* 10- *or* 11-hydroxy-IPR (Figure 4-26, structure I). The complete loss of acetic acid during chromatography, however, strongly supports the identification of this metabolite as 7- *and/or* 10-hydroxy-IPR. The nonderivatized chromatogram contained two peaks, #576N and 593N (Figure 4-24), which gave mass spectra consistent with either one being this primary metabolite.

Peaks # 647A and 651A (Figure 4-25). When scanned, these partially overlapping peaks provided very similar mass spectra, each containing ions of *m/z* 298, 227 $[M-71]^+$, 226 $[M-72]^+$, 199, 198, 171, 170, and 58 (base peak). They are isomers of mass 14 amu greater than that of IPR. Application of the mass spectral information provided above indicates that both metabolites possess an intact 5-[3-dimethylaminopropyl] side-chain and an underivatized aromatic ring. To accommodate all of these observations, these metabolites must both be oxidized products of IPR in which a CH_2 group in the cyclooctyl ring of IPR is replaced with a C=O moiety (Figure 4-26, structure II). The exact locations of these ketone groups are not yet known. In support of this conclusion, the same two peaks are observed in the spectrum of the underivatized metabolites (#583N and 586N; Figure 4-24).

Peaks # 704A, 723A and 772A (Figure 4-25). The metabolites that are acetylated to give these three peaks are minor ones. Comparisons of their mass spectra reveal that they are acetylated derivatives of monooxygenated products of IPR. Their mass spectra are similar, but there are significant differences. Simple perusal of these mass spectral data reveal that the spectra of peaks #723A and 772A are virtually identical, but differ from the spectrum of peak #704A. In particular, the spectra of metabolites #723A and 772A contain relatively abundant fragment ions of *m/z* 229, 228 and 186 which are insignificant in the spectrum of metabolite #704A. In contrast, the spectrum of metabolite #704A contains relatively abundant fragment ions of *m/z* 212, 211, 170 and 144 which are not significant in the spectrum of metabolites #723A and 772A.

These spectra are readily interpreted. All three metabolites retain the 5-[3-dimethylaminopropyl] side-chain, as indicated by the presence of ions *m/z* 271 $[M-71]^+$ and 58 (base peak) in each spectrum. In spectra #723A and 772A, a ketene molecule is expelled from the *m/z* 271 ion, producing the ion *m/z* 229 which fragments further by the loss of a hydrogen atom to give fragment *m/z* 228. This facile expulsion of ketene is characteristic of acetylated phenols (Figure 4-

22a). Appropriate structures for fragment ions, *m/z* 271, 229 and 228 are easily deduced (Figure 4-27). Thus GC peaks #723A and 772A are acetylated derivatives of two isomeric primary metabolites of IPR, each of which is a phenol (Figure 4-26, structure III). To determine the exact locations of these phenolic groups requires further study.

The third metabolite (#704A) is not a phenolic acetate. The presence of the fragment *m/z* 211 in its spectrum is informative. This fragment represents the loss of 60 amu (acetic acid) from the $[M-71]^+$ ion. As discussed above, elimination of acetic acid in this way is characteristic of acetates of cyclic alcohols. It can be concluded that analyte #704A is the acetate of an alicyclic ring monohydroxylated metabolite of IPR. The metabolically introduced hydroxyl group must be located at one of the positions C_6, C_8, C_9 or C_{11} (Figure 4-26, structure I). Its location at the other alicyclic ring positions (C_7, C_{10}) is not likely since spontaneous and complete expulsion of acetic acid during GC-mass spectral analysis of its acetate would have occurred for the reason discussed earlier (section 4.4.1.2).

It is difficult to equate unequivocally peaks in the nonacetylated GC trace with the three peaks just discussed. In Figure 4-24, however, two peaks are present (#593N and 629N) whose mass spectra are consistent with each being an alicyclic hydroxylated derivative of IPR. Both spectra contain a base peak of *m/z* 58, a molecular ion of *m/z* 300, 16 amu greater than that of IPR, and both have fragment ions of *m/z* 282 $[M\text{-}H_2O]^+$. No isolated peaks were detected in the nonacetylated GC trace (Figure 4-24) that corresponded to the acetylated peaks #723A and 772A.

Peaks #953A, 1334A, 1497A, 1514A, 1572A, 1612A and 1869A (Figure 4-25). The EI mass spectra of all of these peaks have the fragment ion of *m/z* 114 as the base peak. As indicated in the mass spectral information of general application provided above, all peaks are derivatives of N-acetylated norIPR.

The mass spectrum of *peak #953A* contains important fragment ions of *m/z* 212 $[M\text{-}100]^+$, 170, 144 (Figure 4-20), 198 $[M\text{-}114]^+$ and 114 (Figure 4-21), and is readily identified as N-acetyl-norIPR, derived from the primary metabolite norIPR [Figure 4-26, structure VIII]. Underivatized norIPR is peak #517N in Figure 4-24.

The mass spectrum of *peak #1334A* indicates that this metabolite has a molecular weight of 370 amu. As with the acetylated metabolite #704A (Figure 4-25), this spectrum contains diagnostic fragment ions that confirm the presence of an acetylated hydroxyl group in the cyclooctyl ring system: a fragment of mass 60 amu (CH_3COOH) is expelled from the molecular ion to produce the major fragment ion of *m/z* 310, and, in a complementary manner, the fragment ion of *m/z* 270 also eliminates an acetic acid molecule in the production of the abundant *m/z* 210 ion. These observations permit the conclusion that the structure of compound #1334A is x-acetoxy-norIPR which was formed by acetylation of the metabolite x-hydroxy-norIPR (Figure 4-26, structure IV) in which x is one of the locations C_6, C_8 C_9 or C_{11}, but not C_7 or C_{10} (contrast peak #625A; Figure 4-25).

The mass spectra of *peaks #1572A and 1612A* are very similar to that of metabolite 1334A, and they are deduced to be isomeric N,O-diacetylated derivatives of cyclooctyl ring-hydroxylated norIPR, differing only in the location of the cyclooctyl ring hydroxyl group.

The component of *peak #1869A* is also isomeric with acetylated metabolites 1334A, 1572A and 1612A, but its mass spectral behavior is clearly different. Compound 1869A is a phenolic acetate; ketene is expelled during its fragmentation (*m/z* 370 → *m/z* 328; *m/z* 270 → *m/z* 228), and there is no indication that a molecule of acetic acid is eliminated. The metabolite from which analyte #1869A is derived is clearly a phenolic derivative of norIPR (Figure 4-26, structure V). While the position occupied by the OH group in the aromatic ring cannot be deduced from the present study, its most probable location is C_2, since the analogous drugs IMI and DMI are readily hydroxylated at C_2 (Rudorfer and Potter, 1985).

In the nonacetylated spectrum, peaks #626N and 634N (Figure 4-24) have mass spectra which indicate that they are both monohydroxylated derivatives of norIPR. It can be deduced that the hydroxyl group is located in the cyclooctyl ring in both metabolites, since each spectrum contains abundant ions of *m/z* 170 and 171, indicative of an unsubstituted aromatic ring, and each spectrum contains an $[M-H_2O]^+$ ion (*m/z* 268) of low abundance. These peaks correspond to two of the three acetylated species 1334A, 1572A, and 1612A. The third nonacetylated metabolite was not detected. The peak in Figure 4-24 that corresponds to peak #1869A is #665N. It is of particular relevance that in the spectrum of 665N the diagnostic fragment ion *m/z* 170, indicative of non-substitution in the aromatic ring, is absent, while an abundant ion 16 amu greater in mass (*m/z* 186) is present.

Three ions of diagnostic value are present in the mass spectrum of *peak #1497A*. These are the ions of *m/z* 114, $[M-114]^+$ and $[M-100]^+$ which have already been identified. Most fragment ions in the EI mass spectrum of this N-acetylated derivative of norIPR are 14 amu greater in mass than those observed in the mass spectrum of acetylated norIPR. This observation can be explained by the presence of a ketone group in the cyclooctyl ring. The conclusion is that compound 1497A is an N-acetylated derivative of norIPR in which one of the ring CH_2 groups has been replaced with a C=O group, and the actual metabolite (Figure 4-26, structure VI) is probably the norIPR homologue of compounds #647A and 651A. Peak #609N

(Figure 4-24) in the GC trace of nonacetylated metabolites presumably corresponds to acetylated peak #1497A.

Peak #1514A is an abundant one in the acetylated metabolite GC trace (Figure 4-25), as is the corresponding peak (#615N) in the GC trace of nonacetylated urine extract. The mass spectrum of peak #1514A is very similar to that of peak #1497A; the metabolites which gave rise to these peaks are readily concluded to be isomeric (Figure 4-26, structure VI), differing only in the position occupied by the introduced ketone group in the cyclooctyl ring. Such a conclusion would be compatible with acetylated metabolites being related in the same manner that metabolites #647A and 651A are related.

Diagnostic ions in the mass spectrum of the nonacetylated metabolite 615N are of *m/z* 284 $[M^{+\cdot}]$, 227 $[M-57]^+$, 226 $[M-58]^+$, 171 and 170. The diagnostic value of these ions has already been discussed.

The fragment ions in the mass spectrum of *peak #1408A* are generally of 14 amu less than those observed in the spectrum of peak 1497A. The structure of the abundant fragment *m/z* 100 in spectrum #1408A has already been identified. A further comparison of spectra #1408A and 1497A indicates conclusively that they are of analogous compounds, and that the former is the N-acetylated derivative of nor-didemethyl-IPR in which a cyclooctyl ring CH_2 group has been oxidized to C=O. This metabolite is therefore 5-aminopropyl-6,7,8,9,10,11-hexahydro-5*H*-cyclooct[*b*]indole-**x**-one (Figure 4-26, structure VII), where **x** is one of positions C_6 to C_{11}. No isolated peak corresponding to #1408A was detected in the GC trace of the nonacetylated metabolites.

This combined gas chromatographic-mass spectrometric study has shown that IPR is metabolized *in vivo* in the rat by three major pathways: alicyclic oxidation to produce a variety of cyclic alcohols and ketones; aromatic oxidation to produce various phenols; and N-demethylation and N,N-didemethylation of the N_5 side-chain. Some metabolites are products of more than one of these metabolic pathways. In total, 14 different metabolites have been characterized.

4.4.2 Biliary excretion study

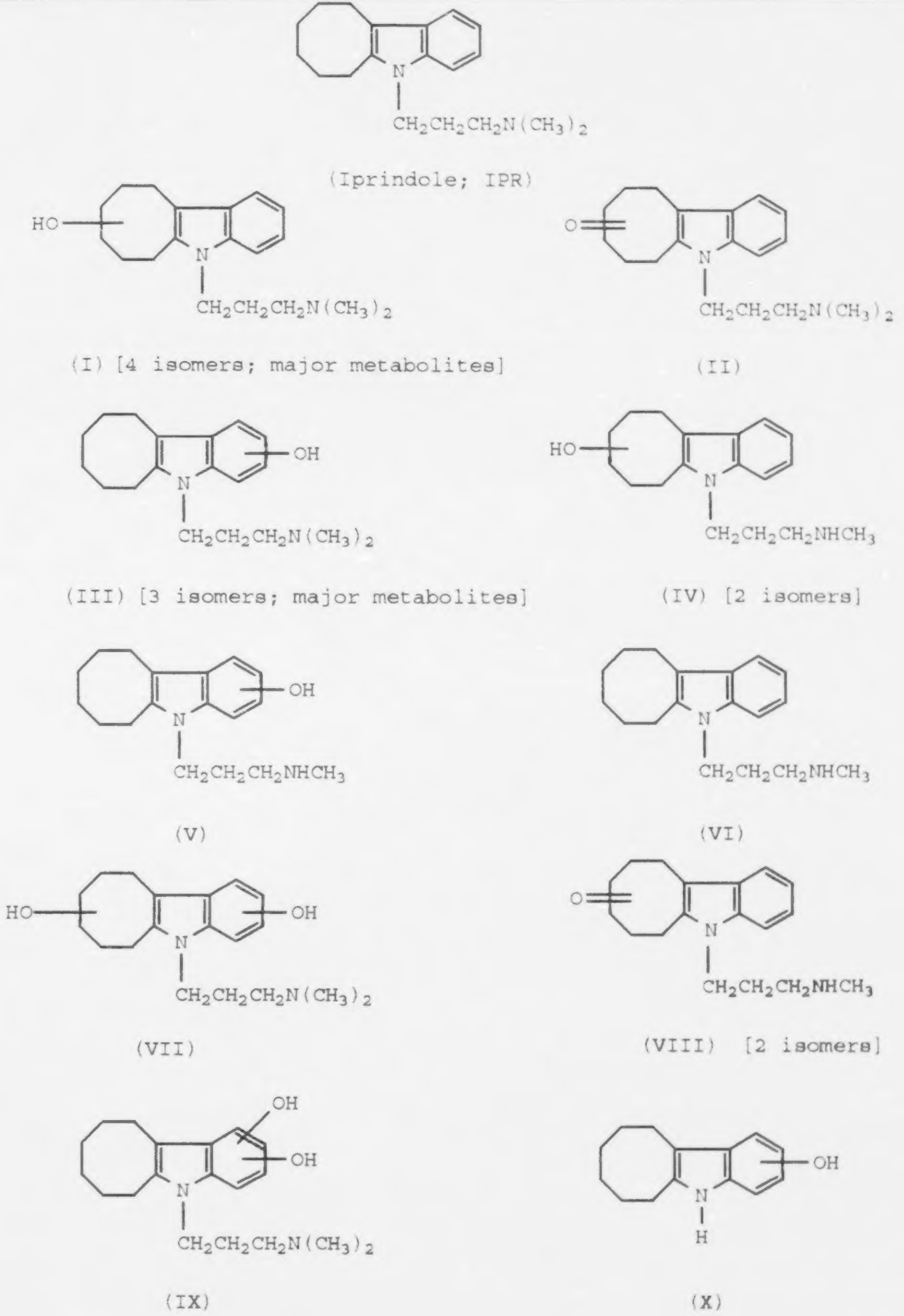
Structures of IPR and its biliary metabolites were determined by interpretation of the fragmentation patterns apparent in each EIMS and by comparing the spectra with those of urinary metabolites (section 4.4.1). In addition to unchanged IPR, 17 metabolites were identified in the bile extract (underivatized and acetylated). The structures of the biliary metabolites of IPR are depicted in Figure 4-28. The major biliary metabolites (from detector response) include isomeric aromatic ring hydroxylated derivatives of IPR as well as alicyclic (cyclooctyl ring) hydroxylated derivatives of IPR and norIPR. In contrast, the major *in vivo* metabolites of IPR, isomeric cyclooctyl ring ketonic derivatives of norIPR (Section 4.4.1), were absent in the bile extract, suggesting that they are excreted mainly *via* kidney in this species. Metabolites # VII, VIII, IX and X (Figure 4-28) were not detected in the urine extract (see section 4.4.1) indicating that these metabolites undergo mainly biliary excretion in the rat.

Three concurrent metabolic pathways were identified for IPR in rat bile, namely alicyclic oxidation, N-dealkylation and aromatic oxidation. The present study indicated that appreciable amounts of IPR metabolites undergo biliary excretion in this species. It is plausible that, like other ADs (e.g. IMI), IPR and some of its hydroxylated metabolites also undergo enterohepatic recirculation. Further work is needed to determine the extent and chemical species involved in enterohepatic recirculation.

4.4.3 In vitro metabolic studies

4.4.3.1 Rat liver microsomes

The structures of the *in vitro* metabolites of IPR were determined by interpreting the EIMS of individual metabolite, as well as by comparing them with those obtained from urinary and biliary metabolites (sections 4.4.1 and 4.4.2). Besides unchanged IPR, 6 metabolites were characterized. The structures of the metabolites are depicted in Figure 4-29. The major *in vitro* metabolite is norIPR.


N
CH$_2$CH$_2$CH$_2$N(CH$_3$)$_2$
HO
N
CH$_2$CH$_2$CH$_2$N(CH$_3$)$_2$
O
N
CH$_2$CH$_2$CH$_2$N(CH$_3$)$_2$
OH
N
CH$_2$CH$_2$CH$_2$N(CH$_3$)$_2$
HO
N
CH$_2$CH$_2$CH$_2$NHCH$_3$
OH
N
CH$_2$CH$_2$CH$_2$NHCH$_3$
N
CH$_2$CH$_2$CH$_2$NHCH$_3$
HO
OH
N
CH$_2$CH$_2$CH$_2$N(CH$_3$)$_2$
O
N
CH$_2$CH$_2$CH$_2$NHCH$_3$
OH
OH
N
CH$_2$CH$_2$CH$_2$N(CH$_3$)$_2$
OH
N
H

(Iprindole; IPR)

(I) [4 isomers; major metabolites]

(II)

(III) [3 isomers; major metabolites]

(IV) [2 isomers]

(V)

(VI)

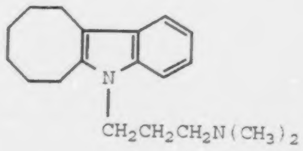
(VII)

(VIII) [2 isomers]

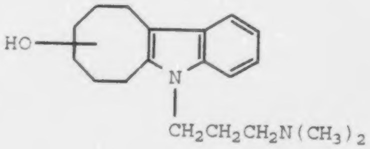
(IX)

(X)

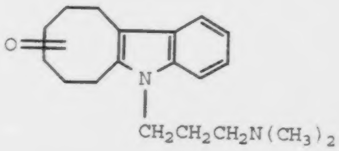
Figure 4-28. Structures of the biliary metabolites of IPR in the rat.


N
CH2CH2CH2N(CH3)2

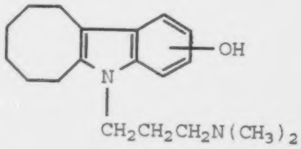
(Iprindole; IPR)


HO
N
CH2CH2CH2N(CH3)2

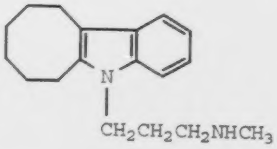
(I)


O
N
CH2CH2CH2N(CH3)2

(II) [2 isomers]


OH
N
CH2CH2CH2N(CH3)2

(III) [2 isomers]


N
CH2CH2CH2NHCH3

(IV)

Figure 4-29. Structures of *in vitro* metabolites of IPR in the rat.

4.5 Studies conducted on NBA

4.5.1 Effects of QND on the metabolism of NBA in the rat[2]

It has been demonstrated by Coutts *et al.*(1978) that *in vivo* metabolism of NBA in rats produced 4-OH-NBA and 4-OH-3-OCH_3-NBA (Figure 4-30). It was reported earlier in this thesis (sections 4.1.3.2 and 4.3.2), that QND pretreatment **increased** the metabolic formation of 2-OH-norTMP and at the same time the formation of 2-OH-TMP was decreased in the rat. For comparison purposes the following study was conducted to delineate the effects of QND pretreatment on the *in vivo* metabolism of NBA in the rat and determine whether QND pretreatment could result in a **increase** of aromatic ring-hydroxylation of other substrates.

Studies on the metabolism of N-alkylated amphetamines in man have shown that the length or bulk of the N-substituent has a profound effect on the amount of administered drug that can be accounted for. With amphetamine (AMP), N-methylamphetamine (NMA), and N-ethylamphetamine (NEA), total recovery of drug as unchanged N-alkylated drug or as AMP was in the 40-78% range; with N-*n*-propylamphetamine (NPA), 27-65% of the dose could be accounted for, but with NBA, N-benzylamphetamine and N-*o*-chlorobenzylamphetamine, 90% or greater of the administered drugs was unaccounted for, even under conditions of acidic urinary pH (Beckett and Shenoy, 1973; Gorrod, 1973). Of particular reference to the current study is NBA, of which only 10% has been accounted for in man, 7% as AMP, and 3% as unmetabolized drug, when subjects were maintained under acidic urinary conditions. Studies on the metabolism of NPA and NBA in rats and man (Coutts *et al.*, 1976; Coutts and Dawson, 1977; Coutts *et al.*, 1978) confirmed that the extent of N-dealkylation to AMP is negligible if urine is not acidified, and also revealed that phenolic metabolites of NPA and NBA (4-hydroxy and 3-methoxy-4-hydroxy derivatives) accounted for additional amounts (up to 17%) of the administered N-alkylamphetamines in man, but most of the drug, especially NBA, remained unaccounted for. With NPA, results from rats and man were comparable as far as the formation of phenolic metabolites were concerned. From this one observation, the rat appears to be a suitable model of metabolic ring hydroxylation of N-alkylamphetamines in man. Previous studies (Coutts *et al.*, 1978) also revealed that NEA, NPA and NBA were each metabolized in the rat to 4-hydroxy and 3-methoxy-4-hydroxy derivatives, the latter *via* catecholamine intermediates, whereas AMP and NMA formed only 4-hydroxylated metabolites. Clearly the length of the amphetamine N-alkyl substituent has a significant effect on the nature and extent of aromatic ring hydroxylation.

---

[2] Dr. F. Malik began the study reported in this chapter but was unable to complete it.

The cytochrome P450 isozyme P450IID6 catalyzes the ring-hydroxylation of many aromatic basic drugs of diverse structures (Brosen, 1990; Robbins and Wedlund, 1990; Nebert *et al.*, 1989; Brosen and Gram, 1989a; Kalow, 1986; Jacqz *et al.*, 1986; Eichelbaum, 1982). In humans, ring hydroxylation catalyzed by this isozyme is strongly inhibited by prior administration of QND, a potent competitive inhibitor of P450IID6 (Ayesh *et al.*, 1991; Boobis et al., 1990; Brosen and Gram, 1989a and 1989b; Kobayashi et al., 1989). Methoxyphenamine (MPA), the ring 2-methoxylated derivative of NMA, undergoes metabolic aromatic hydroxylation catalyzed by cytochrome P450IID6 in human, and this metabolic pathway is inhibited by the prior administration of QND (Muralidharan *et al.*, 1989a). MPA is similarly metabolized by ring hydroxylation in two species of rat [Lewis; Dark Agouti]; again, this metabolic pathway is significantly inhibited by the prior administration of QND (Muralidharan *et al.*, 1989b). The biotransformation of MPA in rat was apparently catalyzed by the P450IID6 isozyme, or a structurally closely related isozyme of the P450IID family. The effect of QND on the metabolism of AMP has only recently been reported (Moody *et al.*, 1990). This investigation was conducted in male Lewis rats, and it was shown that the extensive ring hydroxylation of AMP that occurred in rats receiving no other drugs was greatly reduced by the administration of QND 2h prior to the AMP challenge. The conclusion from these observations was that ring hydroxylation of AMP in Lewis rats was under the influence of cytochrome P450IID6. All studies so far reported on the effect of QND on P450IID6-catalyzed drug oxidative metabolism indicate that it is an effective inhibitor of this metabolic process (Ayesh *et al.*, 1991; Boobis *et al.*, 1990).

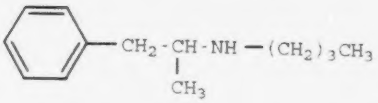
This observed inhibitory effect of QND on the metabolism of AMP and its analog MPA in rat, and the observation that the length of the N-substituent in N-alkylated AMP derivatives greatly influences the nature and extent of metabolic ring hydroxylation prompted the question whether the length of the N-substituent on the AMP nucleus would also have an effect on the ability of QND to inhibit metabolic ring hydroxylation. Thus, a protocol was designed to study the metabolism of (±)-NBA in the rat, in both absence and presence of QND and compare the results with those obtained in sections 4.1.3.2 and 4.3.2.

NBA and its two phenolic metabolites were extracted from hydrolyzed urine samples from drug-treated rats and analyzed by GC-NPD and GC-MS methods. The analytical procedure permitted the simultaneous determinations of NBA and its two phenolic metabolites, 4-OH-NBA and 4-OH-3-OCH_3-NBA (Figure 4-30); structures of these three compounds were confirmed by a comparison of their GC and MS behaviors with those of the synthesized authentic compounds. For quantitation of metabolites, 4-methoxy-NBA (4-OCH_3-NBA) was used as the internal standard.

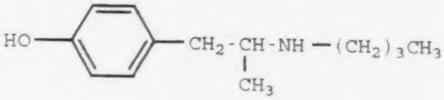
Quantitation of NBA and the two phenolic metabolites in 48h urines collected from rats that had received only NBA showed that 1.1% of the dose was recovered as unchanged NBA, 8.8% of the NBA administered was recovered as 4-OH-NBA, and 2.9% was isolated as 4-OH-3-OCH_3-NBA (total amount recovered 12.8%) (Figure 4-32). These quantities are comparable to those of the corresponding phenolic metabolites isolated in previous studies on NPA metabolism in rats (Coutts *et al.*, 1976) and man (Coutts and Dawson, 1977). Pretreatment of rats with a single dose of QND 1h prior to the administration of NBA altered the amounts of NBA, 4-OH-NBA, and 4-OH-3-OCH_3-NBA recovered to 2.2%, 11.7% and 4.5%, respectively (total amount recovered 18.4%), while three pretreatments with QND were more dramatic in effect, the recovered amounts of NBA, 4-OH-NBA, and 4-OH-3-OCH_3-NBA now being 3.4%, 18.9% and 2.1%, respectively (total amount recovered 24.4%). These results are in distinct contrast with those obtained for AMP. The study by Moody *et al.* (1990) showed that pretreatment of rats with one larger dose of QND (0.102 mmol/kg) 2h prior to the administration of AMP sulfate (15 mg/kg) resulted in a greater than 85% **decrease** in the 48h-urinary excretion of 4-OH-AMP and a greater than 50% increase in the 48h-urinary excretion of unchanged AMP.

QND is a known inhibitor of cytochrome P450IID6-catalyzed drug oxidations, including aromatic oxidation to phenolic metabolites (Boobis *et al.*, 1990). It has been concluded that this cytochrome P450 isozyme is involved in the aromatic 4-hydroxylation of AMP (Moody *et al.*, 1990), and also in the ring oxidation of MPA (Muralidharan *et al.*, 1989b). Since the ring hydroxylation of NBA is significantly enhanced in the presence of QND, one conclusion from the study now reported is that, unlike AMP and MPA, the metabolic oxidation of NBA to phenolic products is **not** catalyzed by P450IID6. It appears from this study that the length of the N-alkyl substituent not only influences the recovery of amphetamines and their metabolites from urine (Beckett and Shenoy, 1973; Gorrod, 1973; Coutts *et al.*, 1978), but also has some influence on the cytochrome P450 isozyme involved in their oxidative metabolism.

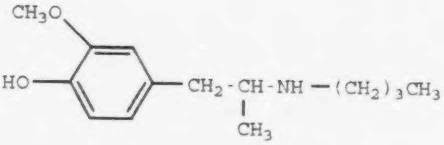
Similarly, it has also been reported earlier in this thesis (sections 4.1.3.2 and 4.3.2) that pretreatment of rats with quinidine prior TMP or norTMP administration significantly **enhanced** the ring hydroxylation of norTMP. One conclusion from these observations is that, as with NBA, the aromatic hydroxylation of norTMP is **not** catalyzed by the cytochrome P450IID6 isozyme.



N-(*n*-butyl)amphetamine [NBA]

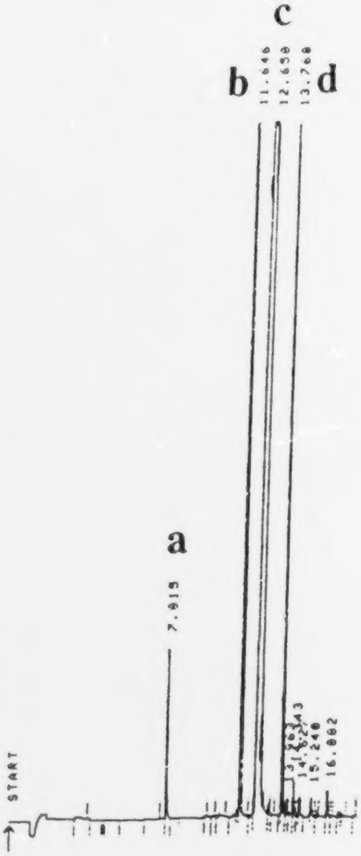


4-OH-NBA



$4\text{-OH-3-OCH}_3\text{-NBA}$

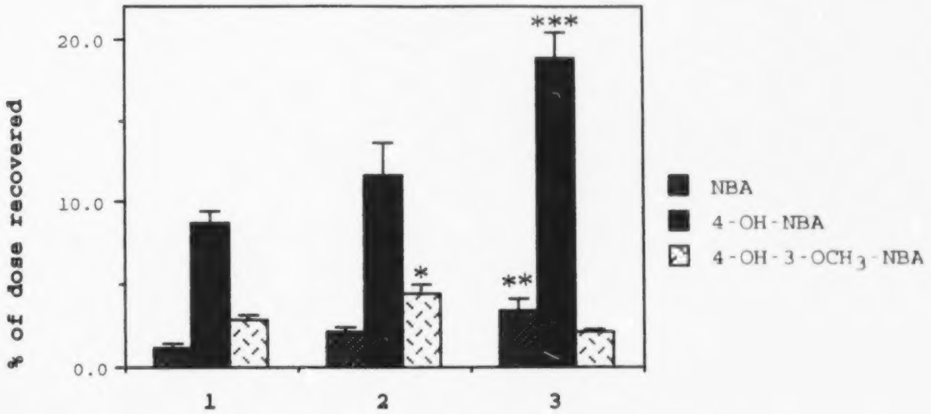
Figure 4-30. Chemical structures of NBA, 4-OH-NBA and $4\text{-OH-3-OCH}_3\text{-NBA}$.



a. NBA
b. 4-OCH_3-NBA
c. 4-OH-NBA
d. 4-OH-3-OCH_3-NBA

Figure 4-31. GC trace of an urine extract from a rat treated with N-(*n*-butyl)amphetamine.

b. is the internal standard.



Phases: 1=NBA; 2=QND(1dose)+NBA; 3=QND(3doses)+NBA

Figure 4-32. Urinary excretion (48h) of N-(*n*-butyl) amphetamine and metabolites in the rat [Mean ± S.E.M.].

*Significantly different from phase 1 and 3 values; **Significantly different from phase 1 value; ***Significantly different from phase 1 and 2 values, $p<0.05$.

4.6 Studies conducted on IMI

4.6.1 Metabolism of IMI in human

The first generation TCA drug IMI has been used effectively for over 30 years and several studies on the analysis of IMI and its metabolites in biological matrices have been reported (Weder and Bickel, 1968; Bickel and Weder, 1968; Crammer et al., 1969; Gram et al., 1983; Maurer and Pfleger, 1984; DeVane and Simpkins, 1985; Potter and Manji, 1990). DeVane and Simpkins (1985) used high performance liquid chromatography to quantify IMI and 3 of its major metabolites (DMI, 2-OH-IMI and 2-OH-DMI) in rat plasma. Because of its sensitivity and relative ease in operation, GC has proven to be a popular analytical procedure for the analysis of IMI and metabolites in biological specimens. Weder and Bickel (1968) identified seven metabolites of IMI in rat tissues (liver, lung, kidney and fat tissues) using a GC-FID. In this study the metabolites were identified by a comparison of their retention times with those of authentic samples of metabolites. One disadvantage of this method is an inability to deduce structures for the separated metabolites. Combined GC-MS is now the method of choice for the rapid determination and structural characterization of drugs and metabolites in biological matrices. In one reported study Maurer and Pfleger (1984) identified IMI and 4 of its *in vivo* metabolites (2-OH-IMI, DMI, 2-OH-DMI, IDB) in human urine using GC-MS technique. The identified metabolites were not quantified. The paper neither described the fragmentation patterns of the individual metabolites nor included a GC trace of separated metabolites.

Most studies (Gram *et al.*, 1983; DeVane and Simpkins, 1985; Potter and Manji, 1990) identified IMI and metabolites in human plasma. Crammer *et al.*(1969) used thin layer chromatography followed by colour tests to identify most of the urinary metabolites in man. But the technique is time-consuming and not sensitive enough for quantitative metabolic studies. Therefore, a computerized GC-MS study was designed to identify and quantify the urinary metabolites of IMI in human after a therapeutic dose. The study was also conducted to determine the contribution of alicyclic oxidation in the total metabolism of IMI.

The urine samples were hydrolyzed enzymatically prior to extraction of IMI and its metabolites and their analysis. The chemical structures of the metabolites were deduced by in-depth interpretation of the fragmentation patterns apparent in each EIMS, as well as by comparisons with the EIMS of some authentic samples of metabolites.

Total ion current chromatograms of the underivatized and acetylated urine extracts are depicted in Figures 4-33 and 4-34 respectively. GC peaks due to IMI and its metabolites were identified in both the traces. Both the underivatized and acetylated urine extracts were analyzed under identical GC-MS conditions. The mass fragmentation pathways of IMI and DMI are depicted in Figure 4-35. Appropriate chemical structures of the isolated metabolites and their EI mass spectra are shown in Figure 4-36. The identities of the underivatized and acetylated metabolites of IMI were deduced by interpretation of the EIMS obtained by scanning each GC peak. Structures of the common and major diagnostic fragment ions of all IMI metabolites are provided in Fig.4-37.

During the interpretation of EI mass spectra of drug and metabolites it was observed that IMI itself and all the metabolites that retained an intact N,N-dimethylpropylamine $[-CH_2CH_2CH_2N(CH_3)_2]$ side chain at N_5 gave rise to mass spectra that contained prominent diagnostic ions of *m/z* $[M-45]^+$, 85 and 58, which are identified in Figure 4-35. The ion of *m/z* 58 is often the base peak in these spectra. For comparison purposes, it may be mentioned here that the spectra of TMP and its metabolites that retained an intact side chain at N_5 also contained this diagnostic ion of *m/z* 58 and in most cases it is the base peak in the spectrum. Similar reasoning allowed the conclusion that mono-N-demethylated metabolites (DMI and derivatives) that possessed a $[-CH_2CH_2CH_2NHCH_3]$ side chain at N_5 may be identified by the presence of diagnostic fragment ions of *m/z* $[M-31]^+$ and *m/z* 71, which are also identified in Figure 4-35.

A comparison of the GC-MS data derived from underivatized and acetylated urine extracts provided information on sites of metabolic oxidation. Thus dealkylation oxidations were readily identified and aromatic and alicyclic oxidations were easily distinguished. The spectra of alicyclic (C_{10} or C_{11}) hydroxylated metabolites usually contain an $[M-18]^+$ fragment ion before chemical derivatization (see section 4.1.1.1). However, when subjected to anhydrous acetylation, these C_{10} or C_{11} hydroxylated metabolites were dehydrated to products that contained a double bond at C_{10}. In contrast, the mass spectra of aromatic (C_2) hydroxylated metabolites did not contain fragment ion resulting from the elimination of water. In addition, aromatic hydroxylated and N-demethylated metabolites were readily acetylated when reacted with acetic anhydride under anhydrous conditions (cf. section 4.1.1.1).

Structure elucidation

IMI (EIMS #1, Figure 4-36) and its metabolites DMI (EIMS #3 and #4; Figure 4-36), 2-OH-IMI (EIMS #11 and 12; Figure 4-36), 2-OH-DMI (EIMS #13 and 14; Figure 4-36) and IDB (EIMS #2; Figure 4-36), were identified by interpreting EI mass spectra of their underivatized and acetylated species by methods similar to those used for TMP metabolites (see section 4.1.1.1) and also by comparison with the spectra of authentic samples of metabolites. The only exception was the mass spectrum of acetylated derivative of DMI (EIMS #4;

Figure 4-36). EIMS #4 contained a $M^{+\cdot}$ of *m/z* 308, while other important fragment ions in the spectrum are of *m/z* 235 $[M-CH_3NHCOCH_3]^+$,208 and 193 (Figure 4-35). The important diagnostic fragment ion present in the spectrum is of *m/z* 114 (Ib) (Figure 4-37), which is absent in the corresponding undervatized species (EIMS #3, Figure 4-36). The fragment ion *m/z* 114 is prominent and often the base peak in the spectra of N-acetylated derivatives of DMI (EIMS #10, 14, 16, 17 and 18). Likewise, the EIMS of norIPR and its derivatives also contained a prominent *m/z* 114 fragment ion in their spectra (section 4.4.1) The mechanism of formation of *m/z* 114 is shown in Figure 4-21 (section 4.4.1). In contrast, acetylated norTMP and its derivatives do not produce EIMS which contain a fragment ion of *m/z* 114 (EIMS #3, Figure 4-3; section 4.1.1.1). Branching in the side chain is apparently a deterrent to this fragmentation pathway.

The structures of remaining metabolites are identified by interpreting their EIMS as below.

Peak 361 [Figure 4-33; t_R 10.23 min]. Inspection of the mass spectrum #5 (Figure 4-36) of this peak revealed its $M^{+\cdot}$ to be of *m/z* 211 (Figure 4-36), which is the base peak in the spectrum. The $M^{+\cdot}$ is 16 amu greater than the $M^{+\cdot}$ (*m/z* 195) of 5H-dibenz[*b,f*]azepine (EIMS #2,Figure 4-36). This was consistent with the mono-oxygenated structure that lacked the N_5 side chain. The metabolite was characterized as 2-hydroxy-5H-dibenz[*b,f*]azepine. Crammer et al. (1969) identified this metabolite in human urine using thin layer chromatography. Further evidence of this conclusion is found in the EIMS #6 of its acetylated derivative.

Peak 379 [Figure 4-34; t_R 11.47 min]. EIMS scanning of this peak produced an EIMS #6 (Figure 4-36) which is virtually identical to that of 2-hydroxy-5H-dibenz[*b,f*]azepine, except for the presence of the expected $M^{+\cdot}$ of *m/z* 253 (Figure 4-36), which is 42 amu ($COCH_2$) greater than that of 2-hydroxy-5H-dibenz[*b,f*]azepine. Absence of dehydration during anhydrous derivatization with acetic anhydride provided evidence of aromatic hydroxylation. These observations indicated that GC peak 456 was the O-acetylated derivative of 2-hydroxy-5H-dibenz[*b,f*]azepine.

Peak 388 [Figure 4-33; t_R 11.07 min]. Interpretation of the EIMS #7 (Figure 4-36) of this GC peak confirmed its identity as 10-OH-IMI. Its $M^{+\cdot}$ was of *m/z* 296 (Figure 4-36), 16 amu greater than that of IMI. The ions of *m/z* 278 $[M-H_2O]^+$ (IIIb) (Figure 4-37), and of *m/z* 251 $[M-45]^+$ (IIb) (Figure 4-37),85 and the base peak of *m/z* 58 (Figure 4-35), indicating an intact $-CH_2CH_2CH_2N(CH_3)_2$ side chain at N_5, are of particular diagnostic value. The presence of fragment ion of *m/z* 278 suggested that the metabolite contained an alicyclic hydroxyl group. Its mass spectrum was identical to that of the authentic sample of 10-OH-IMI. Further confirmation of this structure was obtained from the mass spectrum #8 (Figure 4-36) of its acetylated species.

Peak 337 [Figure 4-34; t_R 10.21 min]. The EIMS #8 (Figure 4-36) of this GC peak contained a $M^{+\cdot}$ of *m/z* 278 (Figure 4-36), which is consistent with the metabolite being 10,11-dehydro-IMI. During anhydrous derivatization with acetic anhydride, C_{10}-C_{11} dehydration occurred. The presence of diagnostic fragment ions of *m/z* 233 $[M-45]^+$ (IV) (Figure 4-37), 85 and the base peak of *m/z* 58 (figure 4-35), is again indicative of an intact N_5 side chain.

Peak 416 [Figure 4-33; t_R 11.90 min]. EIMS scanning of this GC peak showed that its mass spectrum #9 (Figure 4-36) contained diagnostic fragment ions of *m/z* 282 ($M^{+\cdot}$) (Figure 4-36), 264 $[M-H_2O]^+$ (IIIa), 251 $[M-NH_2CH_3]^+$ (IIb), 233 (IV), 211 (IIa) (Figure 4-37), 193, and 71 (Figure 4-35). The presence of the fragment ion of *m/z* 264, suggested that the metabolite contained an alicyclic hydroxyl group. The absence of ions of *m/z* 85 and 58 and the presence of fragment ions of *m/z* 251 and 71 provided evidence of mono-N-demethylation of the N_5 side chain in IMI. These facts are consistent with this metabolite being the result of concurrent alicyclic hydroxylation and N-demethylation. The metabolite was tentatively identified as 10-hydroxy-DMI. IMI metabolism reports rarely mentioned the existence of this species. The identity of this metabolite was further supported by the mass fragmentation patterns of its acetylated derivative, peak 531, Figure 4-34.

Peak 531 [Figure 4-34; t_R 15.40 min]. Inspection of the EI mass spectrum #10 (Figure 4-36) of this peak revealed its $M^{+\cdot}$ to be of *m/z* 306 (Figure 4-36). During derivatization with acetic anhydride, C_{10}-C_{11} dehydration occurred and the N-methylamino group of the side chain was acetylated. Other diagnostic fragment ions present in the mass spectrum of this metabolite are of *m/z* 233 $[M\text{-}HN(COCH_3)CH_3]^+$ (IV), 206 (V) and *m/z* 114 (Ib) (Figure 4-37). The derivatized metabolite is identified as 10,11-dehydro-DMI, the metabolite itself as 10-OH-DMI.

Additional metabolites of IMI were detected only in the acetylated urine extract.

Peak 515 [Figure 4-34; t_R 14.79 min]. This GC peak was of a minor metabolite of IMI. The characteristic fragment ions present in its EIMS #15 (Figure 4-36) were of *m/z* 294 ($M^{+\cdot}$) (Figure 4-36), 235 $[M\text{-}NH_2(COCH_3)]^+$, 208, 193 (Figure 4-35) and 100 (Ia) (Figure 4-37). From the mass spectral fragmentation patterns the metabolite was identified as the acetylated derivative of the N,N-didemethylated metabolite of IMI (DDMI). It is a minor metabolite in this subject. However, it was demonstrated by Gram *et al.*(1983) that plasma levels of DDMI often exceed those of 2-OH-IMI in patients who are poor hydroxylators (PMs) of IMI and DMI.

Peak 716 [Figure 4-34; t_R 17.79 min]. When this broad GC peak was repetitively scanned it was revealed that it contained at least two IMI metabolites. Scanning of the early part of the peak (710) gave an EI mass spectrum #14 (Figure 4-36), and from mass fragmentation pattern it was identified as 2-OH-DMI. Scanning of the latter part of the peak (724) produced a EI mass spectrum #16 (Figure 4-36) which contained fragment ions of *m/z* 324 ($M^{+\cdot}$) (Figure 4-36) and 224 (VI) and 196 $[224\text{-CO}]^+$ (VII) (Figure 4-37). The ions of m/z 324 and 224 are 16 amu (one atom of oxygen) greater than the corresponding fragment ions in the spectrum of N-acetyl-DMI (EIMS #4, Figure 4-36). The presence of diagnostic fragment ion of m/z 114 (Ib) (Figure 4-37) indicated that the metabolite is a derivative of DMI and that metabolic oxidation did not occur in the side chain. There are several ways that one atom of oxygen could be metabolically introduced into the DMI nucleus. Two possibilities could be either N-oxide or epoxide formation. As N-oxides decompose readily in GC-MS conditions, one of the few remaining possibilities is epoxide formation or an expanded ring system (seven membered ring) spontaneously derived from the epoxide. In support of this concept is the observation that naphthalene is metabolically oxidized to 2,3-epoxide which spontaneously rearranges irreversibly to 3-benzoxepin in which the oxygen is contained in a seven membered ring (Testa and Jenner, 1976). The metabolically introduced oxygen atom could be located either at the C_3 or C_2 position. Presumably most of the epoxide formed isomerizes to 2-OH-DMI, while some of it is transformed to an enlarged ring system. This novel metabolite of IMI is tentatively identified as an oxepin analog of DMI.

Peak 743 [Figure 4-34; t_R 20.95 min]. Scanning of this peak produced a EI mass spectrum #17 (Figure 4-36) which contained fragment ions of *m/z* 380 ($M^{+\cdot}$), 280 (VIII), 252 $[280\text{-CO}]^+$ (IX), 210 (X) and the base peak of *m/z* 114 (Ib) (Figure 4-37). The base peak of *m/z* 114 indicated that it is a mono-N-demethylated metabolite of IMI. The spectrum is virtually identical to that of 2-OH-DMI (EIMS #14, Figure 4-36), except for the presence of $M^{+\cdot}$ of *m/z* 380 and fragment ion of *m/z* 280, which are 14 amu greater than the corresponding fragment ions of *m/z* 366 and *m/z* 266 in the spectrum of 2-acetoxy-DMI. This provided evidence of the metabolic introduction of a keto group at an alicyclic carbon (C_{10} or C_{11}) in the ring system. This novel metabolite is identified as either 10 or 11-*oxo*-2-OH-DMI.

Peak 778 [Figure 4-34; t_R 21.98 min]. The EIMS #18 (Figure 4-36) of this peak of low abundance contains a diagnostic fragment ion of *m/z* 114 (Ib) (Figure 4-37), indicative of a mono-N-demethylated side chain. The other fragment ions present are of *m/z* 396 ($M^{+\cdot}$) (Figure 4-36), 354 $[M\text{-}CH_2{=}C{=}O]^+$ (XI) and 296 $[M\text{-}CH_2CH_2N(COCH_3)CH_3]^+$ (XII) and 254 $[354\text{-}CH_2CH_2N(COCH_3)CH_3]^+$ (XIII) (Figure 4-37). The $M^{+\cdot}$ of m/z 396 as well as fragment ion of m/z 296 are 16 amu (1 oxygen atom) greater than corresponding ions of m/z 380 and 280 in the spectrum of 10-*oxo*-2-OH-DMI. The oxygen atom might have been metabolically introduced in the C_{10} or C_{11} position, because this -OH group will not dehydrate during chemical acetylation. From the mass fragmentation patterns this novel metabolite of IMI is tentatively identified as an acetylated derivative of 10-*oxo*-2,11-diOH-DMI or 11-*oxo*-2,10-diOH-DMI.

From mass spectral fragmentation patterns in addition to unchanged IMI, 11 metabolites were characterized. Three minor metabolites are believed to be novel and four had undergone alicyclic oxidation.

From the above GC-MS study four concurrent metabolic pathways have been identified for IMI in man, namely a) aromatic hydroxylation, b) alicyclic oxidation (C_{10} or C_{11}) c) mono and di-N-demethylation of the side chain at N_5 and d) N-dealkylation of the complete side chain at N_5.

4.6.1.1 Quantitation of IMI and metabolites in human urine

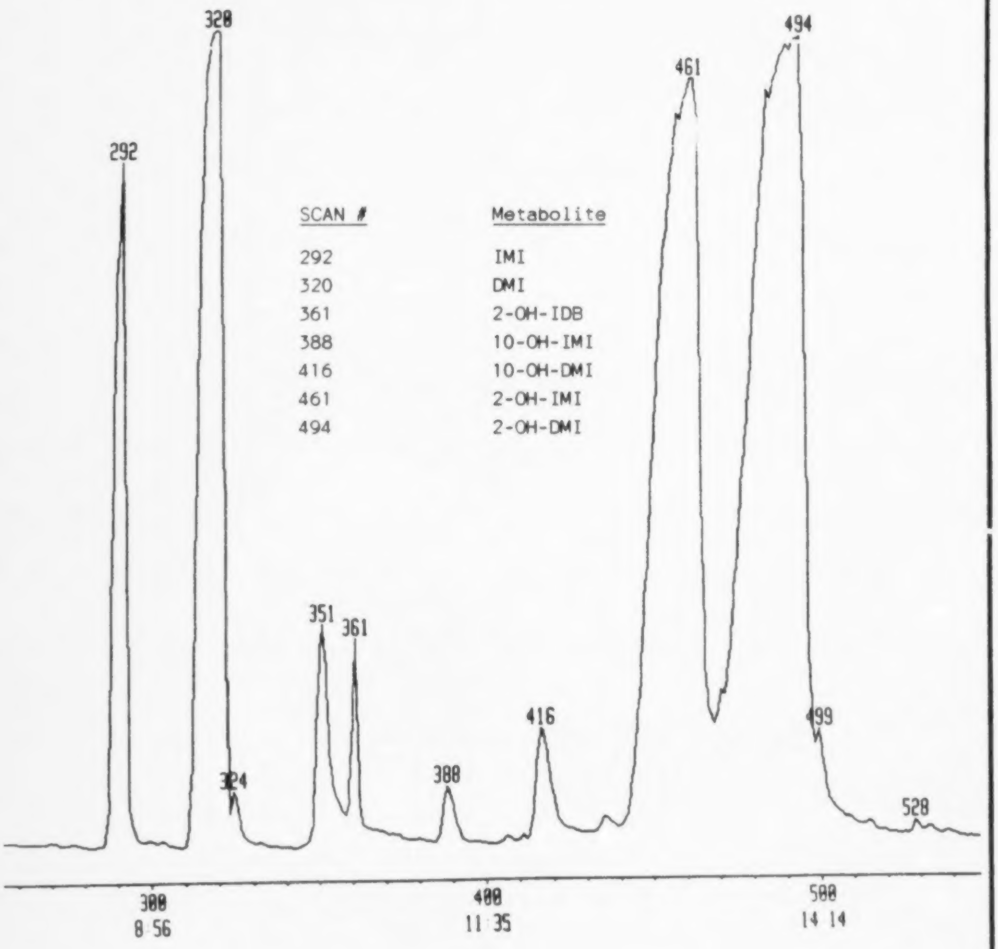
After structural characterization of the urinary metabolites of IMI, quantitation of IMI and its metabolites DMI, 2-OH-IMI and 2-OH-DMI and 10-OH-IMI in the same human urine was performed.

The results from the quantitative study showed that 1.3% of the dose was recovered as IMI, 2.7% as DMI, 0.5% as 10-OH-IMI, 15.1% as 2-OH-IMI and 37.8% was isolated as 2-OH-DMI. This is in agreement with published results (Crammer *et al.*, 1969). Therefore, aromatic hydroxylation is the major metabolic pathway of biotransformation for IMI in man. Because of the non-availability of the authentic samples quantitation of other metabolites was not performed. In the present study 4 alicyclic hydroxylated metabolites of IMI are characterized. The pharmacological properties of some of these metabolites are not known. The quantity of DDMI was negligible in this subject, suggesting that she might be an extensive hydroxylator of IMI and DMI.

4.6.2 Biliary excretion study

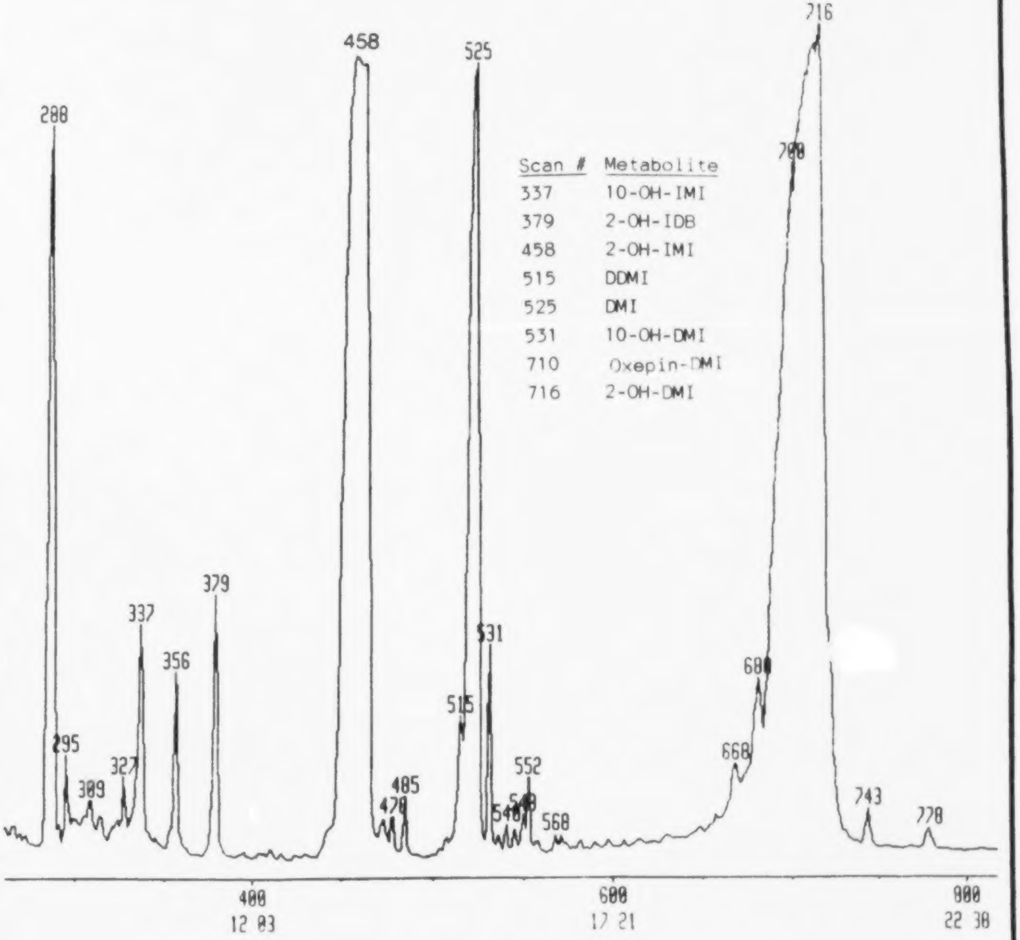
A combined GC-MS technique was employed to determine the structures of IMI and metabolites in enzymatically hydrolyzed bile extract (section 3.13). Interpretation of the mass spectra derived from scanning each GC peak enabled the identification of IMI and 13 of its metabolites in rat bile. Five novel metabolites of IMI were tentatively characterized in rat bile, namely 10-*oxo*-2-OH-IMI, 10-*oxo*-2-OH-DMI, 2,3-diOH-IMI, 2,10-diOH-IMI and 10-OH-IDB, which are consistently formed in small amounts and are not mentioned in any IMI metabolism reports (Bickel and Weder, 1968; Crammer *et al.*, 1969; Erlandsen and Gram, 1982; Gram *et al.*, 1983; DeVane and Simpkins, 1985).

Structures of the biliary metabolites of IMI are depicted in Figure 4-38. The major biliary metabolite of IMI in the rat is 2-OH-DMI. However, 10-OH-DMI, 2-OH-IMI and DMI were also excreted in appreciable amounts.



SCAN #	Metabolite
292	IMI
320	DMI
361	2-OH-IDB
388	10-OH-IMI
416	10-OH-DMI
461	2-OH-IMI
494	2-OH-DMI

Figure 4-33. Total ion current chromatogram of an underivatized urine extract from a female patient dosed po with imipramine.



Scan #	Metabolite
337	10-OH-IMI
379	2-OH-IDB
458	2-OH-IMI
515	DDMI
525	DMI
531	10-OH-DMI
710	Oxepin-DMI
716	2-OH-DMI

Figure 4-34. Total ion current chromatogram of an acetylated urine extract from a female patient dosed po with imipramine.

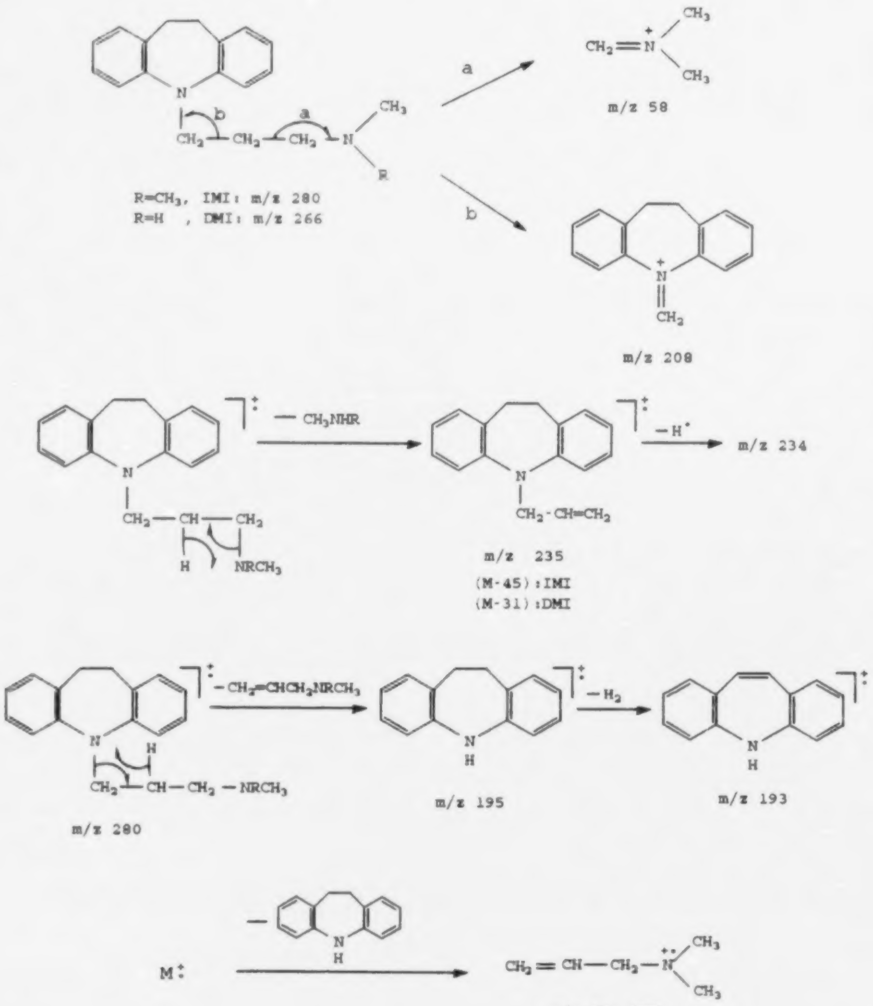

a
b
CH3
R
R=CH3, IMI: m/z 280
R=H , DMI: m/z 266
m/z 58
m/z 208
— CH3NHR
−H·
m/z 234
m/z 235
(M-45):IMI
(M-31):DMI
H
NRCH3
− CH2=CHCH2NRCH3
−H2
m/z 280
m/z 195
m/z 193
M+·
m/z 85: IMI
m/z 71: DMI

Figure 4-35. Mass spectral fragmentation patterns of IMI and DMI.

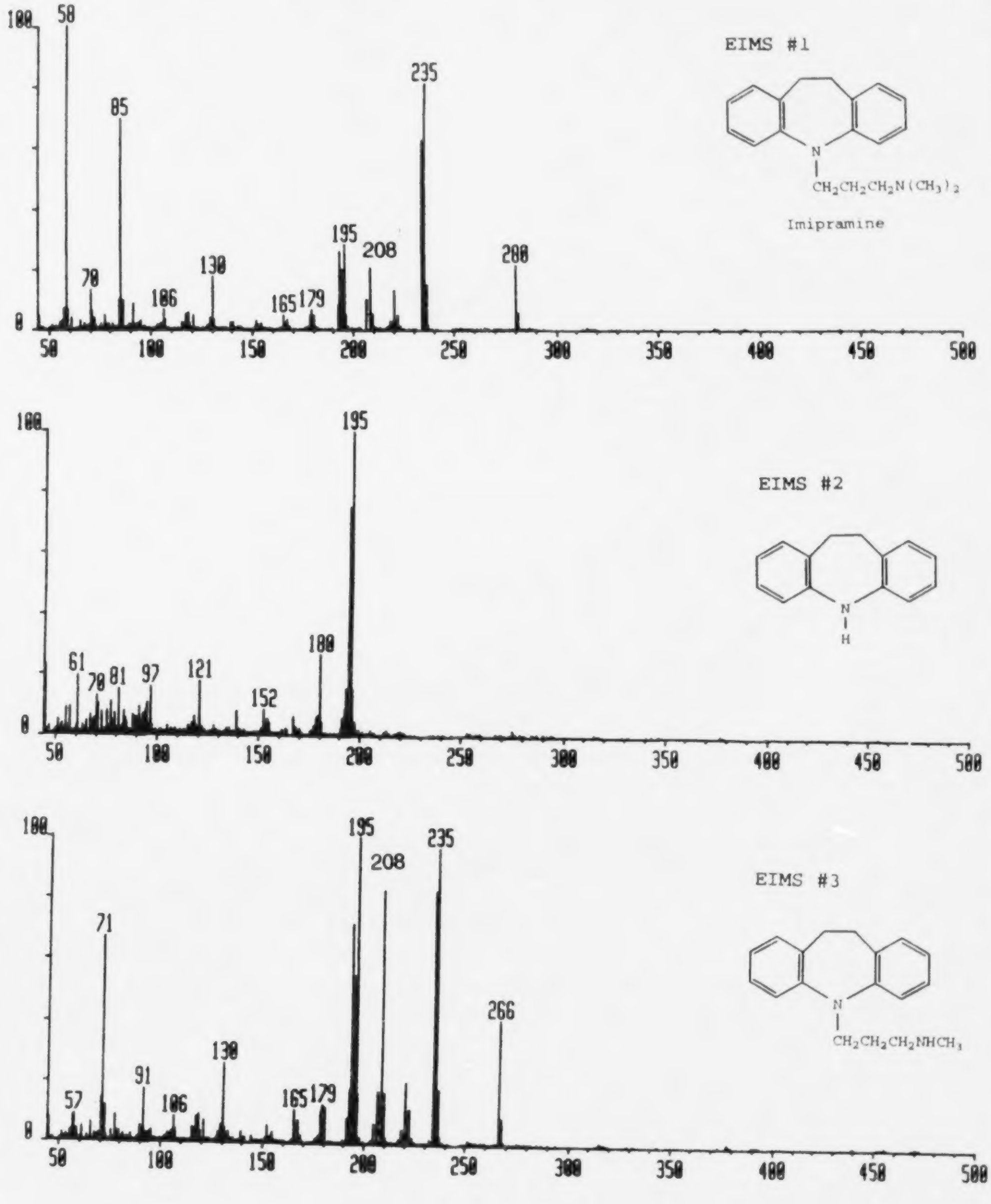


Figure 4-36. Mass spectra and structures of IMI and its underivatized and acetylated metabolites.

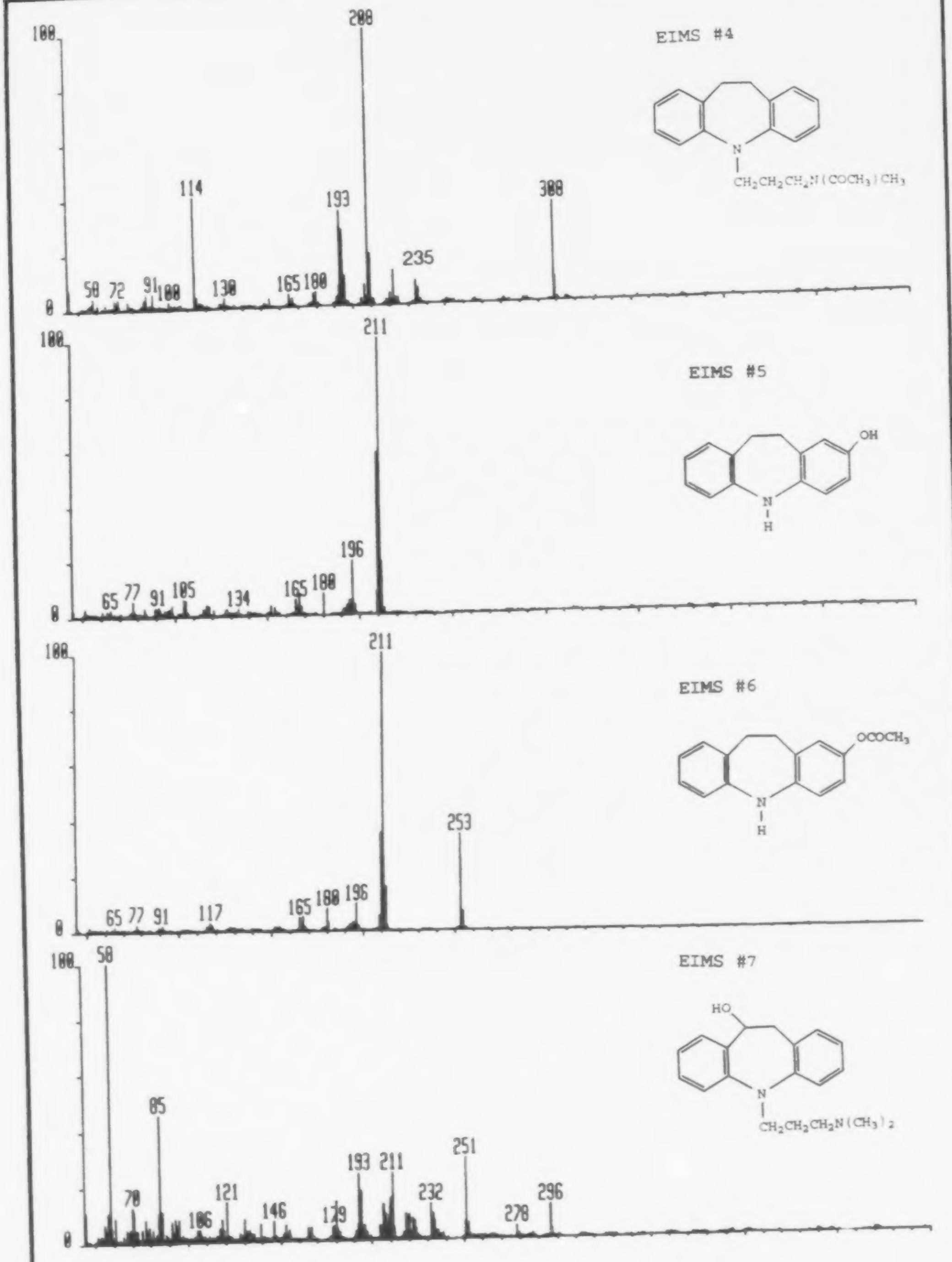


Figure 4-36. Continued

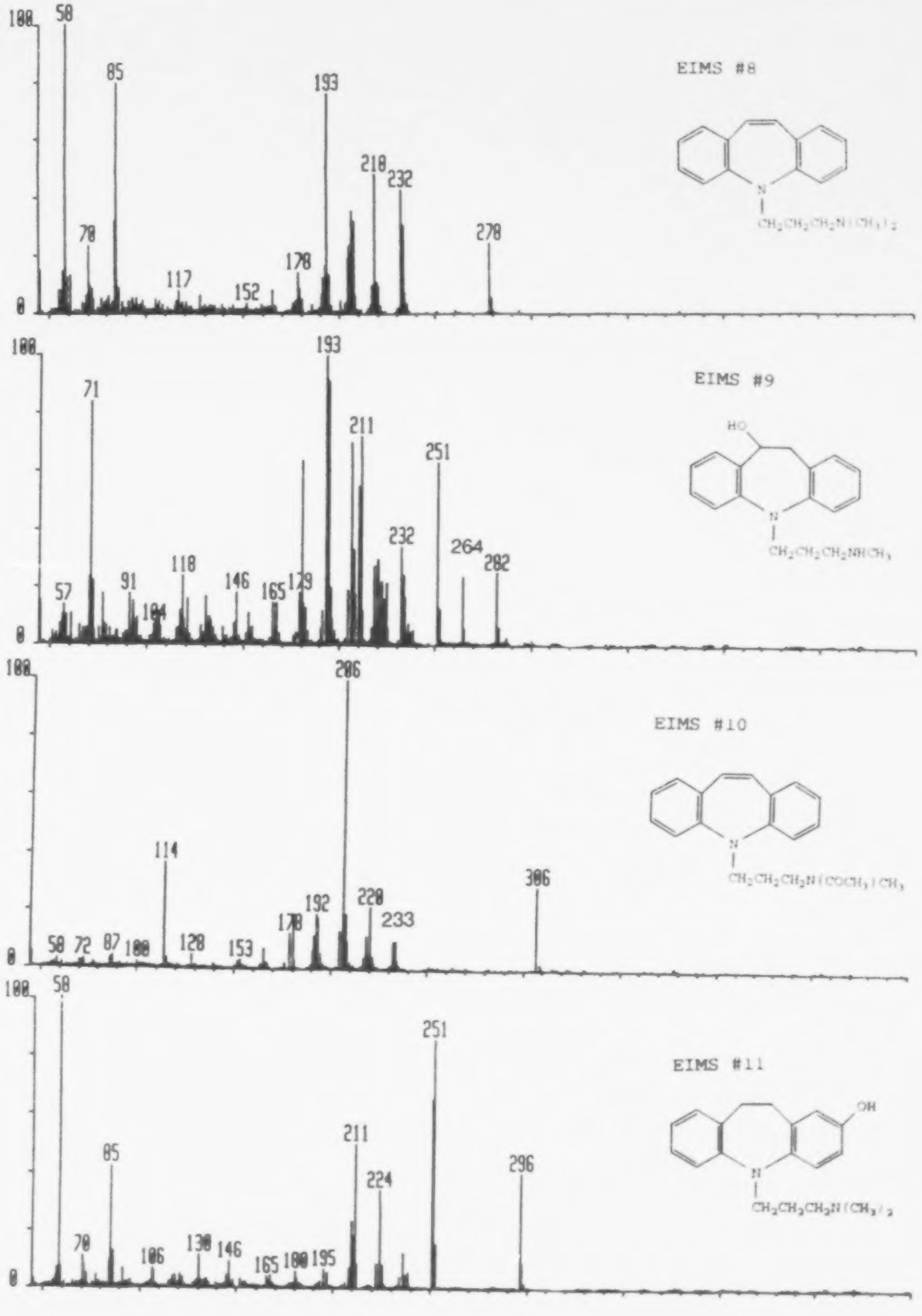


Figure 4-36. Continued

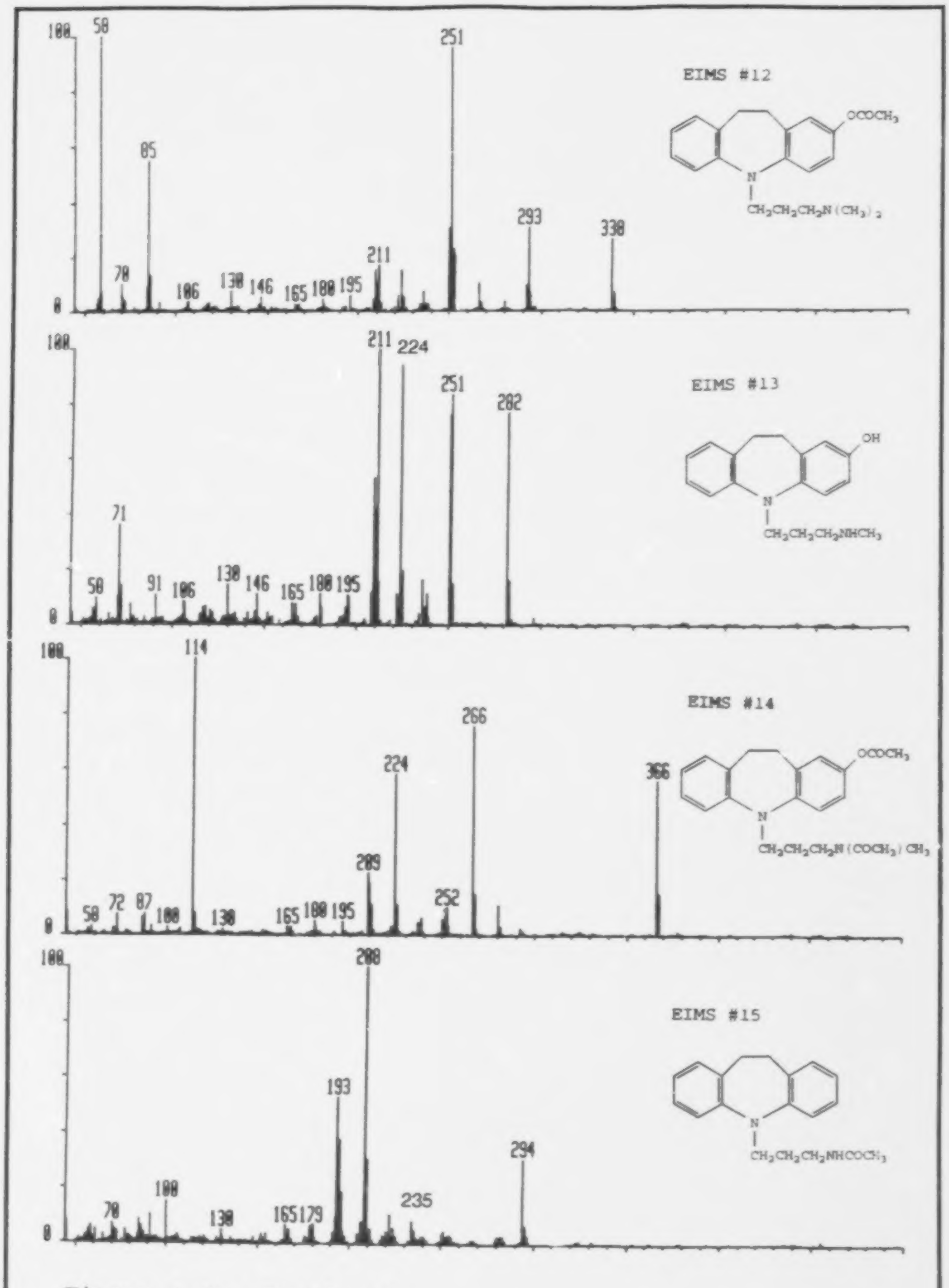


Figure 4-36. Continued

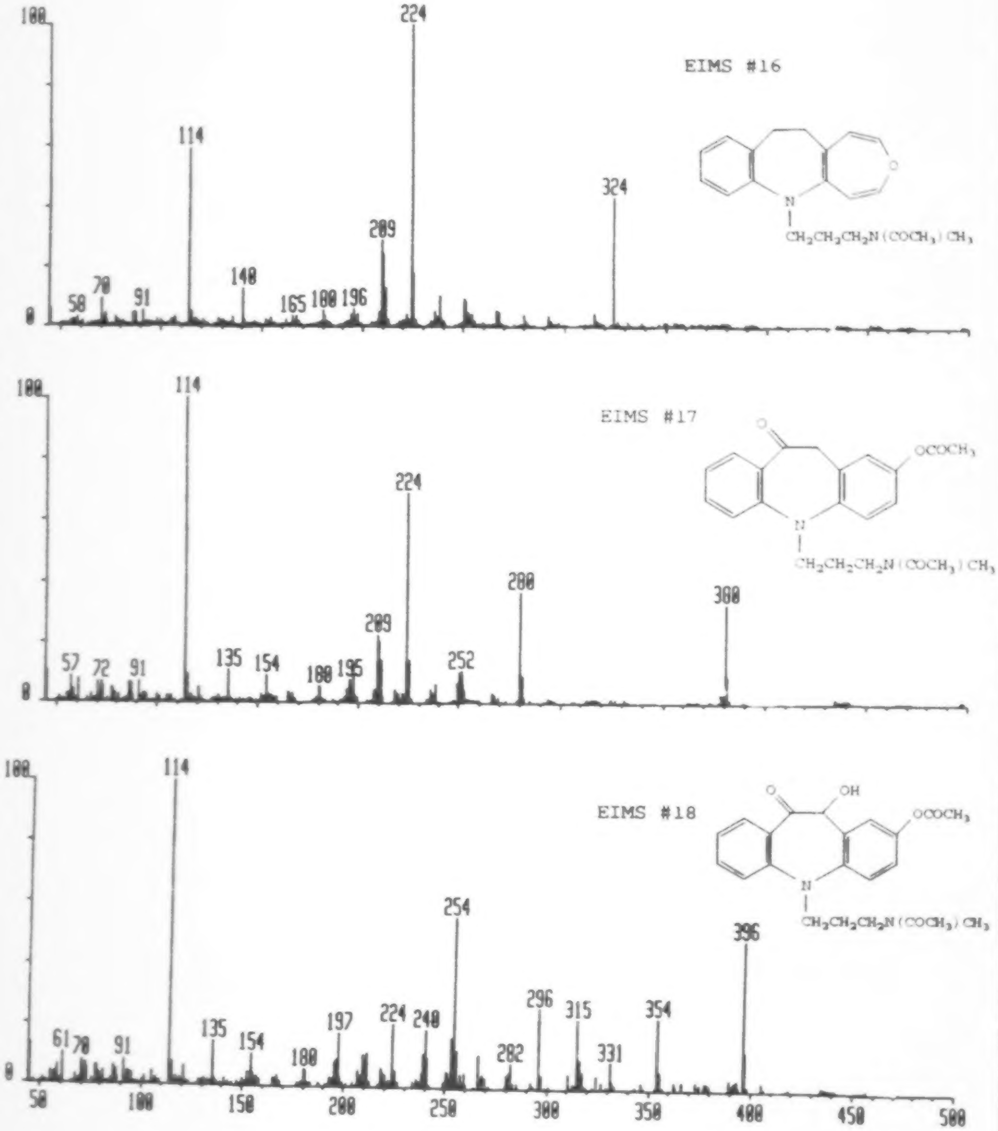
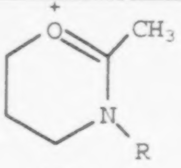
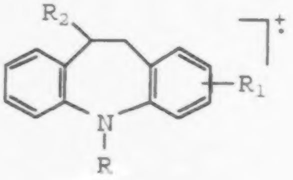


Figure 4-36. Continued



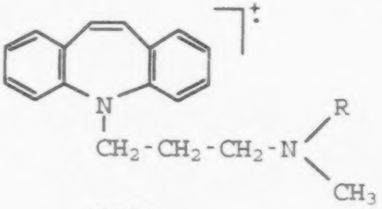
(I)

	R	m/z
a.	H	100
b.	CH_3	114



(II)

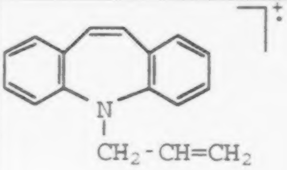
	R	R_1	R_2	m/z
a.	H	H	OH	211
b.	$CH_2CH{=}CH_2$	H	OH	251


+
N
CH2-CH2-CH2-N
R
CH3

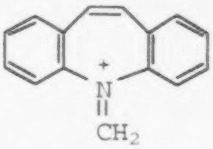
(III)

	R	m/z
a.	H	264
b.	CH_3	278

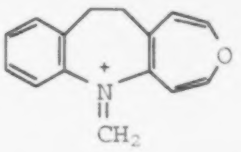
Figure 4-37. Structures of major diagnostic fragment ions of IMI and its metabolites.



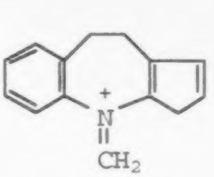
m/z 233; (IV)



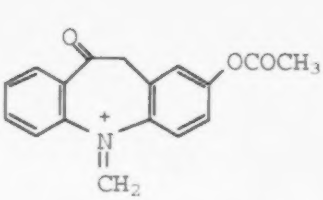
m/z 206; (V)



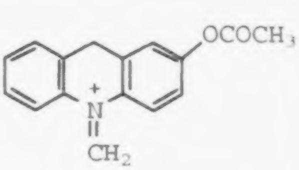
m/z 224; (VI)



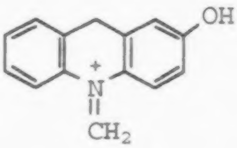
m/z 196; (VII)



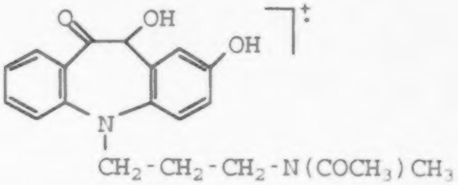
m/z 280; (VIII)



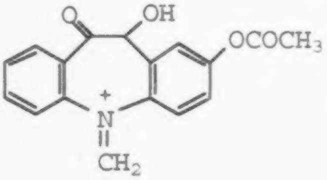
m/z 252; (IX)



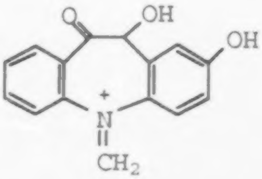
m/z 210; (X)



m/z 354; (XI)



m/z 296; (XII)



m/z 254; (XIII)

Figure 4-37. Continued.

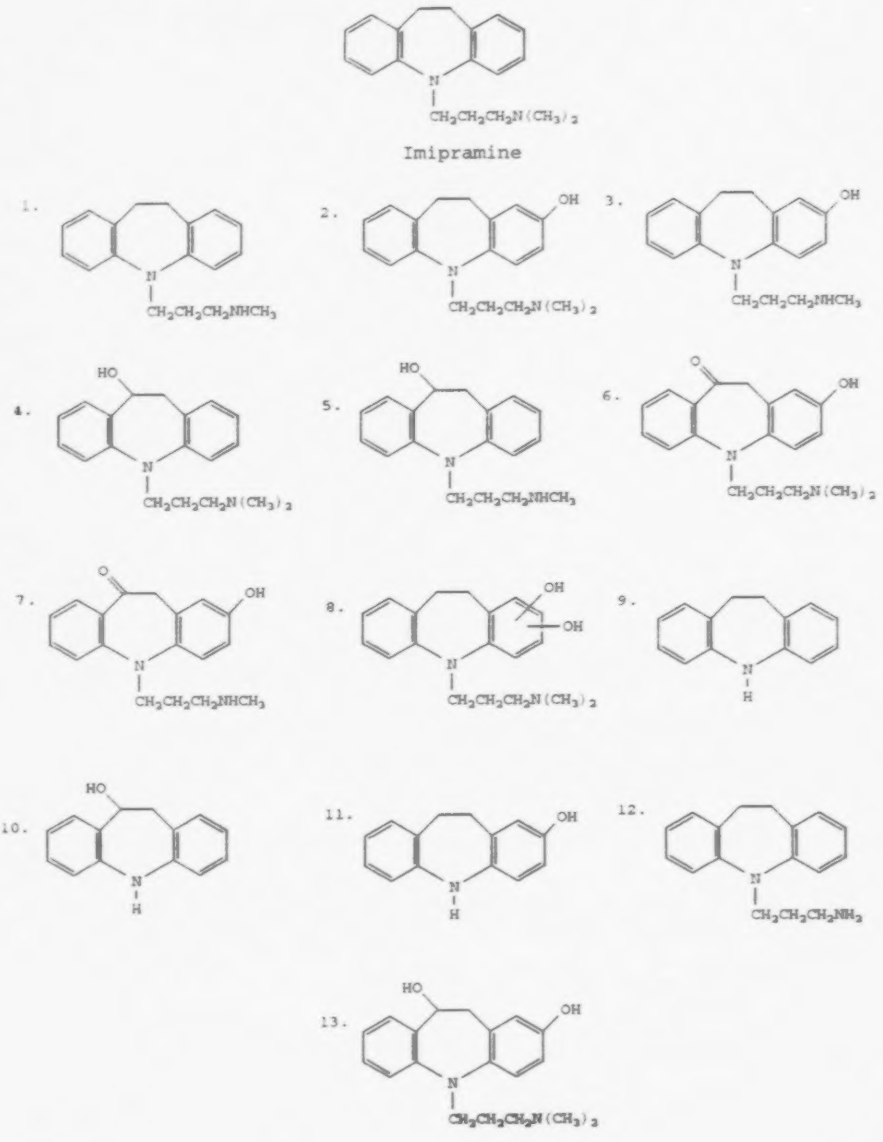

N
CH2CH2CH2N(CH3)2
Imipramine
1.
N
CH2CH2CH2NHCH3
2.
OH
N
CH2CH2CH2N(CH3)2
3.
OH
N
CH2CH2CH2NHCH3
4.
HO
N
CH2CH2CH2N(CH3)2
5.
HO
N
CH2CH2CH2NHCH3
6.
O
OH
N
CH2CH2CH2N(CH3)2
7.
O
OH
N
CH2CH2CH2NHCH3
8.
OH
OH
N
CH2CH2CH2N(CH3)2
9.
N
H
10.
HO
N
H
11.
OH
N
H
12.
N
CH2CH2CH2NH2
13.
HO
OH
N
CH2CH2CH2N(CH3)2

Figure 4-38. Structures of biliary metabolites of IMI in rat.

4.7 Determination of hepatic cytochrome P450IID6 activity in human

Genetic differences in the expression of drug metabolizing enzymes have been recognized as an important determinants in the disposition and responses to certain therapeutic agents. Although patients are not examined for their expression of particular drug metabolism enzymes at the present time, it is important to recognize the degree to which interpatient variability in enzyme expression can affect drug therapy.

A drug metabolism enzyme which has attracted a substantial amount of interest is the P450IID6 enzyme, an isozyme of the cytochrome P450 family which is known to exist in liver. About 5-10% of the Caucasian population exhibits a deficiency in the expression of this isozyme (Eichelbaum *et al.*, 1979; Kalow, 1982; Brosen *et al.*, 1985). A number of diseases, including early onset Parkinsons disease, systemic lupus erythematosis and Balkan nephropathy, have been associated with the absence of P450IID6 isozyme (Meyer *et al.*, 1990; Shahi *et al.*, 1990). Interestingly, this particular drug metabolism enzyme appears to be involved in the oxidative metabolism of over 50 different drugs (Table 1-6, section 1.5.2).

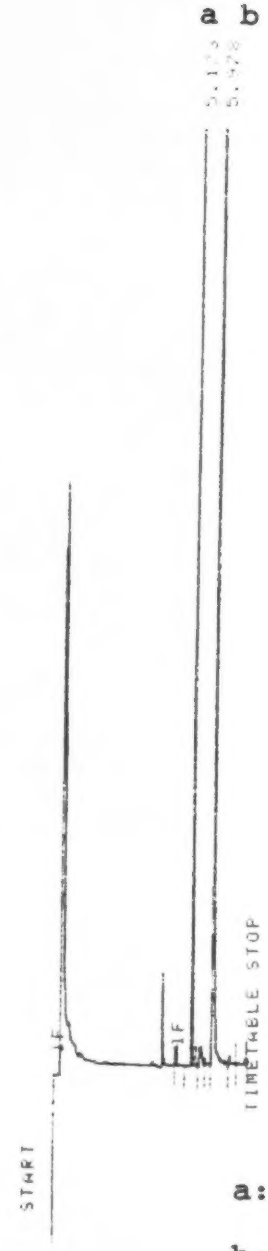
Metabolic oxidations of psychoactive drugs, notably TCAs, are generally catalyzed by the hepatic cytochrome

P450IID6 isozyme (Bertilsson *et al.*, 1980, Brosen *et al.*, 1986a). It has been demonstrated that poor metabolizers (PMs) with respect to cytochrome P450IID6 isozymes activity have a tendency to develop adverse drug side effects (Meyer *et al.*, 1990). It is now known that 10-hydroxylation of AMI and NT as well as 2-hydroxylation of IMI and DMI are all catalyzed by P450IID6, but N-demethylation of AMI to NT and IMI to DMI is regulated by an isozyme other than P450IID6 (Brosen and Gram, 1989b; Brosen, 1990; Ayesh *et al.*, 1991). PMs will clear AMI, NT, IMI, DMI and other drugs whose metabolic oxidation is under the influence of P450IID6 isozyme very much slower than those with a normal complement of the isozyme (EMs), and consequently, PMs suffer from toxic side effects. In contrast, rapid EMs may never attain therapeutic plasma concentrations of the drug when a standard dosage regime is administered to them. It has been estimated that 20-30% of patients belong to these extreme groups (Brosen *et al.*, 1986a).

Phenotyping individuals to determine the hepatic cytochrome P450IID6 activity is based upon the administration of a probe drug (e.g. dextromethorphan, DM) which is metabolized by this isozyme to a product which can be detected in the urine. Although many metabolites of DM have been identified in urine, the O-demethylated product, dextrorphan (DR), represents a major pathway for DM elimination (Koppel et al., 1987). DR is produced predominantly by the cytochrome P450IID6 isozyme and is eliminated in the urine as a glucuronide conjugate (Robbins and Wedlund, 1990). The most common method of obtaining an estimate of P450IID6 enzyme function is the measurement of a metabolic ratio (MR) which has an inverse relation to the activity of this isozyme.

The procedure for phenotyping individuals with the substrate DM is described in section 3.15.

Fifty three adult subjects were enrolled in the study. Only one subject (H052) was a smoker. Except for two (H006 and H007), all subjects were Caucasians. The result of the study is shown in Table 4-2. A typical GC trace of an urine extract from a subject (K019) treated with DM is shown in Figure 4-39. The metabolic ratios (MRs) ranged from 0.004 to 9.6. A total of 13 subjects were identified as PM phenotypes, 39 as EM phenotypes, and one subject's (K017) phenotyping status was undecided. Except for K017, the metabolic ratios in the two urine samples (0-8h and 8-12h) of most of the subjects were very similar. However, the GC trace obtained from an 8-12h urine extract was cleaner and therefore preferred.



a: **Dextromethorphan (DM)**

b: **Dextrorphan (DR)**

Figure 4-39. A typical GC trace of an urine extract from a subject (K019) treated with dextromethorphan.

Table 4-2. Clinical characteristics of subjects and their metabolic ratios (MR).

Subject	Age (yrs)	Sex	Clinical Status	MR(0-8h)	MR(8-12h)	Result
P001	36	F	Psychiatric	0.004	-	EM
P002	39	M	Psychiatric	3.25	-	**PM**
P003	27	F	Psychiatric	1.34	-	**PM**
P004	23	F	Psychiatric	2.43	-	**PM**
S005	28	M	Psychiatric	0.46	-	**PM**
H006	32	F	Healthy	1.04	-	**PM**
H007	40	M	Healthy	*No DM	0.021	EM
H008	28	M	Healthy	0.015	No DM	EM
H009	58	M	Healthy	0.045	0.064	EM
K010	28	F	Healthy	0.029	0.032	EM
K011	35	F	Healthy	0.013	0.013	EM
K012	30	F	Healthy	0.015	0.014	EM
K013	25	F	Healthy	0.240	0.200	EM
K014	29	F	Healthy	0.004	No DM	EM
K015	31	M	Healthy	0.061	0.075	EM
K016	34	M	Healthy	0.016	0.003	EM
K017	24	M	Healthy	0.053	3.020	§UD
K018	30	F	Healthy	0.206	0.125	EM
K019	35	M	Healthy	0.420	0.304	**PM**
K020	40	F	Arthritic	0.009	-	EM
K021	32	F	Arthritic	0.008	-	EM
K022	44	F	Arthritic	0.046	-	EM
K023	32	F	Arthritic	0.009	-	EM
K024	48	F	Arthritic	0.010	-	EM
K025	36	F	Arthritic	0.061	-	EM
K026	32	F	Arthritic	0.079	-	EM
K027	23	F	Arthritic	0.010	-	EM
K028	42	F	Arthritic	0.210	-	EM
K029	34	F	Arthritic	4.990	-	**PM**
K030	25	M	Arthritic	0.008	-	EM
K031	35	F	Arthritic	0.010	-	EM
K032	52	F	Arthritic	0.019	-	EM
K033	34	F	Arthritic	0.125	-	EM
K034	39	F	Arthritic	0.022	-	EM
K035	41	F	Arthritic	0.007	-	EM
K036	48	F	Arthritic	0.005	-	EM
K037	47	F	Arthritic	0.056	-	EM
K038	47	F	Arthritic	0.009	-	EM
K039	36	F	Arthritic	0.005	-	EM
K040	30	F	Arthritic	0.017	-	EM
K041	58	F	Arthritic	1.430	-	**PM**

*No DM=No DM detected; §UD=Undecided

Table 4-2. Continued.

Subject	Age (yrs)	Sex	Clinical Status	MR(0-8h)	MR(8-12h)	Result
K042	62	F	Arthritic	0.008	-	EM
K043	44	F	Arthritic	4.55	-	**PM**
K044	38	F	Arthritic	0.013	-	EM
K045	36	F	Arthritic	0.96	-	**PM**
K046	63	F	Arthritic	0.005	-	EM
K047	36	F	Arthritic	2.510	-	**PM**
K048	29	F	Arthritic	0.005	-	EM
K049	62	F	Arthritic	0.014	-	EM
K050	34	F	Arthritic	0.020	-	EM
P051	28	F	Psychiatric	0.34	-	**PM**
H052	29	M	Healthy	0.012	0.020	EM
H053	25	F	Dental patient	9.6	-	**PM**

Case Studies

The first three of the following case studies were performed in collaboration with Dr. K.F.McKenna, Department of Psychiatry, University of Alberta (McKenna et al., 1991).

Case 1: The subject P004 with a history of depression was referred for evaluation following excessive side effects on a trial of DMI. Her serum level (12h post dose) was noted to be high (480 µg/l) after a normal single po dose of 150 mg DMI. The therapeutic range for DMI is 125-300 µg/l (Orsulak, 1989). She complained of a dry mouth, dizziness and excessive sleepiness. The drug was withdrawn and the patient was phenotyped for hepatic cytochrome P450IID6 activity, as it is known that aromatic hydroxylation of DMI is catalyzed by P450IID6 isozyme (Brosen and Gram, 1988; Brosen and Gram, 1989b). At the time of phenotyping the patient was not taking any medication. Her MR was found to be 2.43, indicating that she was a PM phenotype. DMI was again instituted but the dose was lowered to a single 50 mg po and the serum level was then found to be in the therapeutic range (217 μg/1). Therefore, in this particular case, phenotyping information was very useful for rational drug therapy.

Case 2: Following a single po dose of 25 mg DMI, subject P002 developed marked palpitations and tachycardia. He was instructed to take a second dose and again noticed marked tachycardia 6-8h post dose. The medication was discontinued and was phenotyped for hepatic P450IID6 activity. The phenotyping results clearly showed that he was a PM (MR=3.25).

Case 3: When subject P003 was treated with a single po dose of 75 mg DMI, she suffered from marked cardiovascular side effects, particularly hypotension. Her serum DMI level was 297 μg/1. She was assessed for her hepatic P450IID6 activity and was found to be a PM (MR=1.34). The drug was reinstituted but the dose was reduced to a daily po dose of 50 mg DMI.

Case 4: Subject H053 suffered from methemoglobinemia when she was given prilocaine (a local anesthetic) by her dentist. Presumably this was due to an excessive amount of aromatic amine metabolite(s) in her system. With the thought that she possessed a defective metabolic oxidation profile, she was phenotyped for her hepatic P450IID6 activity. The phenotyping results revealed that she is an extreme PM phenotype. Her MR was 9.6, which is well above the reported range for the MR value of DM (0.0030-5.27) (Henthron *et al.*, 1989).

5. SUMMARY AND CONCLUSIONS

During the course of this thesis research several new GC analytical techniques were developed for the analysis of TMP, IPR, IMI, N-*n*-butylamphetamine, dextromethorphan and several of their metabolites in biological matrices. A GC analytical method was selected because of its reproducibility, sensitivity and the relative ease with which the gas chromatograph can be linked to a mass spectrometer for the unequivocal structural characterization and identification of the compounds of interest and any new metabolites that may be present in the biological fluids and tissues. By interpreting GC-MS data, several novel metabolites of TMP, IPR, and IMI were identified. The anhydrous derivatization technique developed helped to identify site(s) of metabolic oxidation. Thus dealkylation oxidations were readily identified and aromatic and alicyclic oxidations were easily distinguished.

5.1 Studies conducted on TMP

One method of delineating the cytochrome P-450 isozyme(s) that is(are) involved in drug metabolic oxidation reactions is to administer a known inhibitor of metabolism during, or prior to, administration of the drug of interest. QND is a known inhibitor of the hepatic P450IID6 isozyme responsible for the metabolic oxidations of a vast number of drugs, including the structurally similar drugs IMI and DMI.

IPR is also claimed to an inhibitor of metabolic aromatic hydroxylation of amphetamine. In addition, it has also been demonstrated that IPR is an inhibitor of metabolic N-deethylation of fenfluramine. These studies all suggest that IPR could be a potent inhibitor of metabolic aromatic hydroxylation and N-dealkylation of various substrates in rat. Prior to the commencement of drug-drug interaction studies involving TMP in rat, a knowledge of its metabolism in that species was required. To date no comprehensive study has been reported on the metabolism of TMP in the rat. Therefore, a GC-MS study was designed to identify the urinary metabolites of TMP in male Sprague-Dawley rat. The results show that in the rat TMP underwent alicyclic oxidation (C_{10} or C_{11}) in addition to aromatic hydroxylation and N-dealkylation. Besides unchanged TMP, 20 metabolites were characterized by a computerized GC-MS technique. The most abundant metabolites were 2-OH-TMP, 2-OH-norTMP, 10-*oxo*-TMP and 2-OH-10-*oxo*-TMP, indicative of the dominance of aromatic hydroxylation (2-hydroxylation) and, to a lesser extent, alicyclic oxidation (10-oxo formation). The extent of N-demethylation, revealed mainly by the formation of 2-OH-norTMP, was less important than ring oxidation.

In drug-drug interaction studies involving TMP, QND and its diastereoisomer QNN, it has been observed that both QND and QNN significantly inhibited the formation of 2-OH-TMP, but at the same time formation of 2-OH-norTMP was enhanced.

QND had no effect on the formation of alicyclic hydroxylated metabolites, while QNN inhibited this metabolic pathway to a certain extent. These results clearly show that two different isozymes, presumably of the cytochrome P450 family, are involved in the formation of 2-OH-TMP and 2-OH-norTMP.

The metabolite 2-OH-norTMP could be formed from either 2-OH-TMP by N-demethylation or from norTMP by aromatic hydroxylation. To delineate the probable *in vivo* metabolic pathway of formation of 2-OH-norTMP in the rat, two separate studies were conducted. In one study the rats were dosed with norTMP and the urinary metabolites were characterized by a GC-MS method. The second study was performed by treating rats with 2-OH-TMP. The results from the study showed clearly that 2-OH-norTMP was formed metabolically mainly from norTMP by aromatic hydroxylation. This conclusion was also supported by the results from an *in vitro* metabolism study of TMP using fortified rat liver microsomes. This study demonstrated that the precursor for the metabolic formation of 2-OH-norTMP was norTMP.

Therefore, it was concluded that while the formation of 2-OH-TMP is under the influence of P450IID6 isozyme, aromatic hydroxylation of norTMP to form 2-OH-norTMP is not catalyzed by this isozyme.

In a drug-drug interaction study involving TMP and IPR, it was observed that preadministration of IPR to rats that subsequently received TMP resulted in almost complete inhibition of peaks corresponding to 10-*oxo*-TMP and 2-OH-10-*oxo*-TMP. From this, it was immediately apparent that the IPR pretreatment had resulted in almost complete inhibition of alicyclic 10-oxidation of the TMP nucleus. In addition, the urinary excretion of 2-OH-TMP was decreased, as was anticipated, and that of 2-OH-norTMP was increased, which was unexpected. However, the total amount of TMP excreted as 2-OH-TMP and 2-OH-norTMP did not change significantly. These results also demonstrate that the isozyme involved in the 2-hydroxylation of the secondary amine norTMP differs from the one that catalyzes 2-hydroxylation of the tertiary amine TMP.

The results of the biliary excretion study showed that TMP and its alcoholic (10-OH-derivatives) and phenolic (2-OH-derivatives) metabolites could undergo enterohepatic circulation in rat.

Metabolism of TMP in human

In the human TMP is known to undergo aromatic hydroxylation and N-dealkylation, but no alicyclic hydroxylation at the C_{10} position has so far been reported. In this study, in addition to unchanged TMP, ten metabolites were identified. Three of them were novel, of which two were the result of alicyclic hydroxylation at the C_{10} position (10-OH-TMP and 10-OH-norTMP) and one apparently resulted from the *in vivo* acetylation of 2-OH-N,N-didesmethyl-TMP.

5.2 Studies conducted on IPR

In a reported metabolism study on IPR in various species (Sisenwine *et al.*, 1979), only five metabolites were tentatively identified, although the presence of additional metabolites was indicated by thin layer chromatographic analysis. In the present study fourteen IPR metabolites were isolated and separated by GC-MS analysis. In their study, Sisenwine *et al.*(1979) were unable to confirm the presence of phenolic metabolites or products of N,N-didemethylation of IPR. In the study now reported, one N,N-didemethylated and three phenolic metabolites were isolated although none was a major metabolic product. This finding clearly contrasts with results of TMP's and IMI's metabolism where aromatic ring oxidation is a dominant biotransformation pathway.

Whereas IPR strongly inhibits the deethylation of fenfluramine, its own N-demethylation is a very important metabolic pathway in rats. Eight of the fourteen metabolites, including the two major acetylated metabolites, were N-demethylated products.

While little aromatic hydroxylation of IPR occurs in rats, alicyclic hydroxylation of IPR is a dominant metabolic reaction. Five isomeric alicyclic alcohols were produced and they were identified after chemical acetylation. It is quite possible that oxidation occurs to some extent at all available positions in the cyclooctyl ring system. In some instances, the alcohols are further oxidized to ketones. Five ketones were also identified; the ring locations of the ketone groups are not known. Only one metabolite that was not ring-oxidized, *viz.* norIPR, was isolated. In summary, six of the fourteen isolated metabolites retain an intact 5-[3-dimethylaminopropyl] side-chain, and eight are products of N-demethylation, one of which is N,N-didemethylated. Based on the GC trace areas of their acetylated derivatives, the major metabolites of IPR in the rat are isomeric cyclooctyl ring ketonic derivatives of the N-monodemethylated drug. The same conclusion was derived by Sisenwine *et al.*(1979). The pharmacological properties of these ketones are unknown.

In a biliary excretion study, in addition to unchanged IPR, seventeen metabolites were identified by the GC-MS method. The major biliary metabolites (from relative detector responses) include isomeric aromatic ring hydroxylated derivatives of IPR as well as alicyclic (cyclooctyl ring) hydroxylated derivatives of IPR and norIPR. In contrast, the major *in vivo* metabolites of IPR, isomeric cyclooctyl ring ketonic derivatives of norIPR, were absent in bile extract, suggesting that they are excreted mainly *via* kidney in this species. Some metabolites, *viz.* x,y-diOH-IPR (x=alicyclic, y=aromatic), x-*oxo*-IPR, 2,3-diOH-IPR, and y-OH-6,7,8,9,10,11-hexahydro-5H-cyclooct[b]indole, were absent in the urine extract, indicating that these metabolites undergo mainly biliary excretion in the rat.

5.3 Studies conducted on NBA

This study was conducted to determine whether the cytochrome P450IID6 isozyme was involved in the aromatic hydroxylation of NBA, since it was known that aromatic hydroxylation of amphetamine (AMP) and its analog methoxyphenamine (MPA) is catalyzed by P450IID6 isozyme. It was of prime interest to determine whether the length of the N-alkyl substituent of amphetamines had any influence on the cytochrome P450 isozyme involved in their oxidative metabolism as well as their recovery from urine. QND is a known inhibitor of P450IID6-catalyzed drug oxidations, including aromatic oxidations to phenolic metabolites. Since the ring hydroxylation of NBA was significantly enhanced in the presence of QND, one conclusion from this study was that, unlike AMP and MPA, the metabolic ring oxidation of NBA to phenolic products is not catalyzed by P450IID6 isozyme.

5.4 Studies conducted on IMI

This brief study was conducted on one human to delineate the contribution of alicyclic oxidation in the total metabolism of IMI, as well as to determine the urinary excretion of N,N-didesmethyl-IMI (DDMI) in this subject. In one study Gram *et al.*(1983) demonstrated that poor hydroxylators of IMI and DMI had appreciable plasma levels of DDMI, often exceeding those of 2-OH-IMI.

In the present study eleven metabolites of IMI were characterized; of these four had undergone alicyclic (C_{10} or C_{11}) oxidation, and three are believed to be novel. One observation from this study was that these alicyclic hydroxylated metabolites are formed consistently in small amounts in man and are rarely mentioned in IMI metabolism reports. Another observation from this study was that DDMI is a minor metabolite of IMI in this subject. Presumably she was an extensive hydroxylator of IMI and DMI, consequently quantitative analysis of her urine extract yielded major contributions from 2-OH-IMI and 2-OH-DMI and only minor levels of DDMI.

5.5 Phenotyping experiment

The effect of polymorphism on the expression of the cytochrome P450IID6 isozyme and its possible impact on adverse drug effects, drug removal from the market place and altered drug dosage requirements of certain therapeutic agents has attracted the attention of researchers, the pharmaceutical industry and the regulatory bodies world-wide.

The case studies described in section 4.7 illustrate the clinical relevance of phenotyping patients prior to their receiving antidepressant drugs. As the hydroxylated metabolites of ADs have potent neurochemical effects, with both toxic and therapeutic potential, the pharmacodynamic profiles in PMs would be expected to be different than those in EMs. In addition to identifying PM phenotypes, phenotyping may also prevent prolonged therapeutic trials with sub-therapeutic drug levels in rapid EMs. It can help cut health care costs by allowing the clinician to arrive more quickly at the optimal dosage of some drugs, reducing the length of time to a beneficial response. Phenotyping should thus be introduced into the clinical routine for a rational drug therapy.

6. FUTURE PERSPECTIVES

1. TMP possesses a chiral center and is administered as its racemate. The antidepressant property appears to reside in the (+)-enantiomer [or its metabolites(s)]. Surprisingly, the (-)-isomer is said to have a depressant action (Bowman and Rand, 1980; Reynolds, 1982). All TMP metabolites that retained the chiral center will also be racemates. Proportions of contributing enantiomers are not known. Further studies on this topic are needed to know the enantiomeric composition of these chiral metabolites.

2. Metabolites of TMP, IPR and IMI undergo extensive biliary excretion in rat. These metabolites might undergo enterohepatic circulation. A study could be designed to identify the metabolites which are involved in the enterohepatic circulation.

3. In *in vivo* metabolism studies of TMP in rat it was observed that both QND and QNN enhanced the metabolic formation of 2-OH-norTMP. This observation indicated that P450IID6 isozyme is not involved in the formation of 2-OH-norTMP. It would be interesting to find out which isozyme system is catalyzing this metabolic pathway.

4. It was also observed in the present study that both QND and QNN significantly decreased the metabolic formation of 2-OH-TMP in male Sprague Dawley rats. This observation

suggests that the P450IID6 isozyme catalyzes the formation of 2-OH-TMP. Studies could be designed to investigate the metabolism of TMP in Dark Agouti rats, which are claimed to be models of human PM phenotypes of DQN/SPT, and compare the results with those obtained in male Sprague-Dawley or Lewis rats before and after QND treatment.

5. Alicyclic hydroxylation of TMP was completely inhibited by IPR pretreatment in rat and this metabolic pathway was virtually unaffected by QND pretreatment. This observation indicated that an isozyme other than P450IID6 is involved in catalyzing this metabolic pathway. Further studies are needed to delineate the isozyme responsible for the formation of alicyclic hydroxylated metabolites (10-*oxo*-TMP and 2-OH-10-*oxo*-TMP) of TMP in male Sprague-Dawley rats.

6. It would also be interesting to delineate the effects of IPR pretreatment on the 4-hydroxylation (alicyclic oxidation) of DQN, 10-hydroxylation of AMI and NT in male Sprague-Dawley rats. These metabolic pathways are catalyzed by the P450IID6 isozyme. If IPR does not inhibit P450IID6, 4-OH-DQN, 10-OH-AMI and 10-OH-NT should proceed in the presence of IPR.

7. There is a paucity of information on the metabolism of IPR in humans. Further studies are needed to learn about its biotransformation pathways in humans.

8. IPR inhibits the metabolic N-deethylation of fenfluramine in rat. Studies could be designed to determine the effects of IPR pretreatment on the metabolism of various N-alkylated analogs of amphetamine (e.g. NEA, NPA and NBA) as well as lidocaine and other N-ethyl secondary amine substrates.

9. TMP is a widely used antidepressant drug in Canada. It is claimed that the diverse pharmacological properties of TMP may be associated with its various individual metabolites. It could be hypothesized that its side effects may also reside in individual metabolites. Above all, its mechanism of action is yet to be fully understood. Studies could be designed in future to delineate the effects of low doses of QND, QNN or IPR on the metabolism of TMP in humans. There are clinical implications to the findings of such studies. The resultant change in the metabolic profile of TMP in human could result in a dramatically altered therapeutic and/or side effect profile.

BIBLIOGRAPHY

Abernethy D.R., Greenblatt D.J. and Shader R.I. *Trimipramine kinetics and absolute bioavailability: use of gas-liquid chromatography with nitrogen-phosphorus detection.* Clin. Pharmacol. Therap. **1984**; 35:348-353.

Al-Badr A.A. *Trimipramine maleate.* In: Analytical Profiles of Drug Substances. Florey K., ed. Academic Press Inc., **1983**;vol. 12: 683-712.

Alpers H.S. and Himwich H.E. *An in vitro study of the effects of tricyclic antidepressant drugs on the accumulation of ^{14}C-serotonin by rabbit brain.* Biol. Psychiatry. **1969**; 1:81-85.

Alvarez F.J., Casas E., Franganillo A. and Velasco, A. *Effects of antidepressants on histamine H2 receptors in rat isolated uterus.* J. Pharm. Sci. **1986**; 17: 351-354.

Assalian P., Rosengarten M.D. and Philips R. *A comparative trial of the antidepressant, anxiolytic and cardiovascular effects of trimipramine and doxepine in depressed hospitalized patients.* J. Clin. Psychiat. **1985**;46:90-94.

Axelrod J. *Noradrenaline: Fate and control of its biosynthesis.* Science. **1971**; 173: 598-606.

Ayesh R., Dawling S., Hayler A., Oates N.S., Cholerton B., Idle J.R. and Smith R.L. *Comparative effects of the diastereoisomers, quinine and quinidine in producing phenocopy debrisoquine poor metabolisers (PMs) in healthy volunteers.* Chirality. **1991**; 3: 14-18.

Ayesh R., Idle J.R., Ritchie J.C., Crothers et al., *Metabolic oxidation phenotypes as markers for susceptibility to lung cancer.* Nature. **1984**; 311: 169-170.

Baier-Weber B, Prox A, Wachsmuth H and Breyer-Pfaff U. *Glucuronides of hydroxylated metabolites of amitriptyline and nortriptyline isolated from rat bile.* Drug Metab. Dispos. **1988**; 16: 490-496.

Baker G.B., Coutts R.T. and LeGatt D.F. *Gas chromatographic analysis of amines in biological systems.* In: Analysis of Biogenic amines. Baker G.B. and Coutts R.T. eds, Elsevier, Amsterdam; **1982**; pp. 109-128.

Beckett A.H. and Brookes L.G. *The absorption and urinary excretion in man of fenfluramine and its main metabolite.* J. Pharm. Pharmac. **1967**; 19: 41S-52S.

Beckett A.H. and Shenoy E.V.B. *The effect of N-alkyl chain length and stereochemistry on the absorption, metabolism and urinary excretion of N-alkylamphetamines in man.* J. Pharm. Pharmacol. **1973**; 25: 793-799.

Bend J.R., Hook G.E.R. and Gram T.E. *Characterization of lung microsomes as related to drug metabolism.* Drug Metab. Disp. **1973**; 1: 358-367.

Bertilsson L., Alvan G., von Bahr C., Lind M., Mellstrom B., Sawe J., Schulz H and Sjoqvist F. *Active metabolites of antidepressants: novel aspects of hydroxylation and demethylation in man.* In: Clinical Pharmacology in Psychiatry. Neuroleptic and Antidepressant Research. Usdin E., Dahl S.G., Gram L.F. and Lingjaerde O., eds. Macmillan, London. **1981**; 161-169.

Bertilsson L., Asberg M. and Thoren P. *Differential effects of clomipramine and nortrityline on cerebrospinal fluid metabolites of serotonin and noradrenaline in depression.* Eur. J. Clin. Pharmacol. **1974**; 7: 365-368.

Bertilsson L., *Mellstrom B. and Sjoqvist F. Pronounced inhibition of noradrenaline uptake by 10-hydroxymetabolites of nortriptyline.* Life Sci. **1979**; 25: 1285-1292.

Bertilssson L., Eichelbaum M., Mellstrom B, Sawe J., Schulz H-U and Sjoqvist F. *Nortriptyline and antipyrine clearance in relation to debrisoquine hydroxylation in man.* Life Sci. **1980**; 27: 1673-1677.

Bickel M.H. and Baggiolini M. *The metabolism of imipramine and its metabolites by rat liver microsomes.* Biochem. Pharmacol. **1966**; 15: 1155-1169.

Bickel M.H. and Minder R. *Metabolism and biliary excretion of the lipophilic drug molecules, imipramine and desmethylimipramine in the rat-Experiments in vivo and with isolated perfused liver.* Biochem. Pharmacol. **1970**; 19: 2425-2435.

Bickel M.H. and Weder H.J. *The total fate of a drug: Kinetics of distribution, excretion, and formation of 14 metabolites in rats treated with imipramine.* Arch. Int. Pharmacodyn. **1968**; 173: 433-463.

Blau K. and King G.S. Handbook of Derivatives for Chromatography. Blau K and King G.S. eds., Heyden, London. **1978**; pp. 104-151.

Boobis A.R., Murray S., Kahn G.C., Robertz G.M. and Davies D.S. *Substrate specificity of the form of cytochrome*

P450 catalyzing the 4-hydroxylation of debrisoquine in man. Mol. Pharmacol. **1983**; 23: 474-481.

Boobis A.R., Seddon C.E. and Davies D.S. *Bufuralol 1'-hydroxylase activity of the rat: Strain differences and the effects of inhibitors.* Biochem. Pharmacol. **1986**; 35: 2961-2965.

Boobis A.R., Sesardic D., Murray B.P., Edwards R.J.,Singleton A.M., Rich K.J., Murray S., De La Torre R., Segura J., Pelkonem O., Pasanem M., Kobayashi S., Zhi-Guang T. and Davies D.S. *Species variation in the response of the cytochrome P450-dependent monooxygenase system to inducers and inhibitors.* Xenobiotica. **1990**; 20: 1139-1161.

Bowman W.C. and Rand M.J. In Textbook of Pharmacology, 2nd edition. Blackwell Scientific Publications, London. **1980**; pp.15.16.

Brinn R., Brosen K., Gram L.F., Haghfelt and Otton S.V. *Sparteine oxidation is practically abolished in quinidine-treated patients.* Br. J. Clin. Pharmacol. **1986**; 22: 194-197.

Brodie B.B. *Of mice, microsomes and man.* Pharmacologist. **1964**; 6: 12-26.

Brodie B.B., Axelrod J., Cooper J.R. Gaudette L., LaDu B.N., Mitoma C. and Udenfriend S. *Detoxication of drugs and other foreign compounds by liver microsomes.* Science. **1955**; 121: 603-604.

Brosen K. and Gram L.F. *Clinical significance of the sparteine/debrisoquine oxidation polymorphism.* Eur. J. Clin. Pharmacol. **1989**a; 36: 537-547.

Brosen K. and Gram L.F. *First-pass metabolism of imipramine and desipramine: Impact of the sparteine oxidation phenotype.* Clin. Pharmacol. Ther. **1988**; 43: 400-406.

Brosen K. and Gram L.F. *Quinidine inhibits the 2-hydroxylation of imipramine and desipramine but not the demethylation of imipramine.* Eur. J. Clin. Pharmacol. **1989**b; 37: 155-160.

Brosen K. Gram L.F., Klysner R., Otton S.V., Bech P. and Bertilsson L. *Steady-state concentrations of imipramine and its metabolites in relation to the sparteine/debrisoquine polymorphism.* Eur. J. Clin. Pharmacol. **1986**a; 30: 679-684.

Brosen K. Gram L.F., Klysner R., Otton S.V., Bech P. *Steady-state levels of imipramine and its metabolites: significance of dose-dependent kinetics.* Eur. J. Clin. Pharmacol. **1986**b; 30: 43-49.

Brosen K. *Recent developments in hepatic drug oxidation: Implication for clinical pharmacokinetics.* Clin. Pharmacokinet. **1990**; 18: 220-239.

Brosen K., Gram L.F., Haghfelt T and Berrilsson L. *Extensive metabolizers of debrisoquine become poor metabolizers during quinidine treatment.* Pharmacol. Toxicol. **1987**; 60: 312-314.

Brosen K., Otton S.V. and Gram L.F. *Sparteine oxidation polymorphism in Denmark.* Acta Pharmac. Tox. **1985**; 57: 357-360.

Brosen K., Zeugin T. and Meyer U.A. *Role of P450IID6, the target of the sparteine-debrisoquin oxidation* *polymorphism, in the metabolism of imipramine.* Clin. Pharmacol. Ther. **1991**; 49: 609-617.

Budzikiewicz H, Djerassi C. and Williams D.H. Mass Spectrometry of Organic Compounds. Holden-Day, San Francisco. **1967**; pp 468-471.

Caccia S and Garattini S. *Formation of active metabolites of psychotropic drugs. An updated review of their significance.* Clin. Pharmacokinet. **1990**; 18: 434-459.

Caille G., De Montigny C. and Besner J-G. *Quantitation of iprindole in plasma by glc.* Biopharmaceutics and Drug Disposition. **1982**; 3: 11-17.

Carrington R. and Frigerio A. *Mass fragmentation in drug research.* Drug Metab. Rev. **1977**; 6: 243-282.

Cassidy S. and Henry J. *Fatal toxicity of antidepressant drugs in overdose.* Br. Med. J. **1987**; 295: 1021-1024.

Colvin M., Brunderett R.B., Kan M.N., Jardie I and Fenselau C. *Alkylating properties of phosporamide mustard.* Cancer Res. **1976**; 36: 1121-1126.

Cone E.J. *Analysis of drugs in biological samples by combined gas chromatography-mass spectrometry.* In: Pharmaceutical Analysis. Modern Methods. Munson J.W. ed., Marcel Dekker Inc., New York. **1984**; pp. 143-227.

Conney A.H. *Pharmacological implications of microsomal enzyme induction.* Pharmacol. Rev. **1967**; 19: 317-366.

Conti A and Bickel M.H. *History of drug metabolism: Discoveries of the major pathways in the 19th century.* Drug Metab. Rev. **1977**; 6: 1-50.

Cooper T.B. *Gas-Liquid chromatography of antidepressant, antipsychotic, and benzodiazepine drugs in plasma and tissues.* In: Neuromethods 10: Analysis of psychiatric drugs. Boulton A.A., Baker G.B. and Coutts R.T. eds., Humana Press, Clifton, New Jersey. **1988**; pp 65-98.

Coutts R.T. *Cytochrome P450IIC9.* Personal communications; **1991**b.

Coutts R.T. *Cytochromes P450.* Personal communications; **1991a**.

Coutts R.T. *Trimipramine.* Personal communications; **1989**.

Coutts R.T. and Baker G.B. *Gas Chromatography.* In: Handbook of Neurochemistry: Experimental Neurochemistry. Second edition, Lajtha A ed. **1982**; pp.429-446.

Coutts R.T. and Dawson G.W. *Urinary excretion of phenolic metabolites of N-(n-propyl)amphetamine in man.* Res. Commun. Chem. Pathol. Pharmacol. **1977**; 17:349-352.

Coutts R.T. and Jones G.R. *Significance of analytical techniques in drug metabolism studies.* In: Concepts in Drug Metabolism, Part A. Jenner P. and Testa B. eds. Marcel Dekker, New York. **1980**; pp 1-51.

Coutts R.T., Dawson G.W. and Jones G.R. *In vivo metabolism of N-alkylamphetamines in the rat-The effect of N-alkyl chain length on oxidation of the aromatic ring.* Res. Commun. Chem. Pathol. Pharmacol. **1978**; 22:589-592.

Coutts R.T., Dawson G.W., Kazakoff C.W. and Wong J.Y. *In vivo phenolic metabolites of N-alkylamphetamines in the rat. Evidence in favour of catechol formation.* Drug Metab. Dispos. **1976**; 4: 256-261.

Coutts R.T., Prelusky D.B. and Jones G.R. *The effects of cofactor and species differences on the in vitro metabolism of propiophenone and phenylacetone.* Can. J. Physiol. Pharmacol. **1981**; 59: 195-201.

Cowan D.A., Ozkirimli S. and Beckett, A.H. *The identification and determination of some metabolites of trimipramine.* Acta Pharm. Suec. **1988**; 25: 141-150.

Crammer J.L., Scott B. and Rolfe B. *Metabolism of ^{14}C-imipramine: II. Urinary metabolites in man.* Psychopharmacologia. **1969**; 15: 207-225.

Dallner G., Siekevitz P and Palade G.E. *Biogenesis of endoplasmic reticulum membranes. I. Structural and chemical differentiation in rat hepatocyte.* J. Cell. Biol. **1966**; 30: 73-96.

Dayer P., Desmeules J., Leeman T. and Striberni R. *Bioactivation of the narcotic drug codeine in human liver is mediated by the polymorphic monooxygenase catalyzing debrisoquine 4-hydroxylation.* Biochem Biophys. Res. Commun. **1988**; 152: 411-416.

de Montigny C., Elie R. and Caille G. *Rapid response to the addition of lithium in iprindole-resistant unipolar depression: a pilot study.* Am. J. Psychiat. **1985**; 142: 220-223.

Dencker H., Dencker S.J., Green A. and Nagy A. *Intestinal absorption, demethylation, and enterohepatic circulation of imipramine.* Clin. Pharmacol. Ther. **1976**; 19: 584-586.

DeVane C.L. *Cyclic antidepressants.* In: Applied Pharmacokinetics: Principles of Therapeutic Drug

Monitoring. Second edition, Evans W.E., Schentag J.J. and Jusko W.J. eds, **1986**; 852-907.

Devane C.L. and Simpkins J.W. *Pharmacokinetics of imipramine and its major metabolites in pregnant rats and their fetuses following a single dose.* Drug Metab. Dispos. **1985**; 13: 438-442.

Dollery C.T., Fraser H.S., Mucklow J.C. and Bulpitt C.J. *Contribution of environmental factors to variability in human drug metabolism.* Drug Metab. Rev. **1979**; 9: 207-220.

Drayer D.E. *Pharmacologically active drug metabolites: Therapeutic and toxic activities, plasma and urine data in man, accumulation in renal failure.* Clin. Pharmacokinet. **1976**; 1: 426-443.

Edwards D.J., Sorisio D.A. and Sedlock M.L. *Decrease in tyrosine and p-hydroxyphenylglycol caused by various antidepressants.* Biochem. Pharmacol. **1988**; 37: 2069-2075.

Eichelbaum M., Mineshita S., Ohnhaus E.E. and Zekorn. *The influence of enzyme induction on polymorphic sparteine oxidation.* Br. J. Clin. Pharmacol. **1986**; 22: 49-53.

Eichelbaum M., Spannbrucker N., Steinche B. and Dengler H.J. *Defective N-oxidation of sparteine in man. A new pharmacologic defect.* Eur. J. Clin. Pharmacol. **1979**; 16:183-187.

Erlandsen E.J. and Gram L.F. *Imipramine kinetics in the single pass rat liver perfusion model.* Acta Pharmacol. et toxicol. **1982**; 50: 137-147.

Ettre L.S. and Horvath C. *Foundation of modern liquid chromatography.* Anal. Chem. **1975**; 47: 422A-447A.

Evans D.A.P, Mahgoub A., Sloan T.P., Idle J.R. and Smith R.L. *A family and population study of genetic polymorphism of debrisoquine oxidation in a British population.* J. Med. Genet. **1980**; 17: 102-105.

Faigle J.W. and Dieterle W. *The metabolism and pharmacokinetics of clomipramine (Anafranil).* J. Inter. Med. Res. **1973**; 1: 281-290.

Fraser A.D., Isner A.F. and Perry R.A. *Distribution of trimipramine and its major metabolites in a fatal overdose case.* J. Anal. Toxicol. **1987a**; 11: 168-178.

Freeman J.J. and Sulser F. *Iprindole-amphetamine interactions in the rat: the role of aromatic hydroxylation of amphetamine in its mode of action.* J. Pharmacol. Exp. Ther. **1972**; 183: 307-315.

Fuller R.and Hemrick-Luecke S. *Long-lasting depletion of striatal dopamine by a single injection of amphetamine in iprindole treated rats.* Science. **1980**; 209: 305-307.

Ganes D.A. and Midha K.K. *Identification of in vivo acetylation pathway for N-dealkylated metabolites of doxylamine in humans.* Xenobiotica. **1987**; 17: 993-999.

Gex-Fabry M., Balant-Gorgia A.E., Balant L.P. and Garrone G. Clomipramine metabolism: *Model-Based analysis of variability factors from drug monitoring data.* Clin. Pharmacokinet. **1990**; 19: 241-255.

Gibson G.G. and Skett P. In: Introduction to drug metabolism. Chapman and Hall,New York. **1986**.

Gillette J.R. *Techniques for studying drug metabolism in vitro.* In: Fundamentals of Drug Metabolism and Drug Disposition. La Du B.N., Mandel H.G. and Way E.L., eds. The Williams and Wilkins Company, Baltimore. **1971**; pp.400-418.

Gillette J.R., Mitchell J.R. and Brodie B.B. *Biochemical mechanism of drug toxicity.* Ann. Rev. Pharmacol. **1974**; 14: 271-288.

Glassman A.H., Schildkraut J.J, Orsulak P.J., Cooper T.B., Kufper D.J., Shader R.I.,Davis J.M., Carroll B., Perel J.M., Klerman G.I. and Greenblatt D.J. *Tricyclic antidepressants- Blood level measurements and clinical outcome*: An APA Task Force Report. Am. J. Psychiatry. **1985**; 142: 155-162.

Gluckman M.I. and Baum T. *The pharmacology of iprindole, a new antidepressant.* Psychopharmacologia (Berlin). **1969**; 15: 169-185.

Gonzalez F.J. *Molecular genetics of the P-450 superfamily.* Pharmac. Ther. **1990**; 45: 1-38.

Gonzalez F.J., Matsunaga T., Nagata K., Meyer U.A., Nebert D.W., Pastewka J., Kozak C.A., Gilllette J., Gelboin H.V. and Hardwick J.P. *Debrisoquine 4-hydroxylase: Characterization of a new P450 gene subfamily, regulation, chromosomal mapping, and molecular analysis of the DA rat Polymorphism.* DNA. **1987**; 6: 149-161.

Gonzalez F.J., Skoda R., Kimura S., Umeno M., Zanger U.M., Nebert D.W., Gelboin H.V., Hardwick J.P. and Meyer U.A. *Characterization of the common genetic defect in humans* *deficient in debrisoquine metabolism.* Nature. **1988**; 331: 442-446.

Gorrod J W. *The metabolism and excretion of amphetamines in man.* In: Frontiers in Catecholamine Research. Uddin E. and Synder S.H. eds., Pergamon Press, New York. **1973**; pp 940-945.

Gram L.F. *Metabolism of tricyclic antidepressants: A review.* Dan. Med. Bull. **1974**; 21: 218-231.

Gram L.F., Bjerre M., Kragh-Sorensen P., Kvinesdal B., Molin J. and Reisby N. *Imipramine metabolites in blood of patients during therapy and after overdose.* Clin. Pharmacol. Ther. **1983**; 33: 335-342.

Green A.L., O'Grady J.E. and Vass M. *The effect of some tricyclic antidepressants on the inhibition of mouse brain monoamine oxidase in vivo by phenelzine.* J. Pharm. Pharmacol. **1989**; 41: 50-51.

Guengerich F.P. *Molecular advances for the cytochrome P-450 superfamily.* Trends Pharmacol. Sci. **1991**; 12: 281-283.

Guengerich F.P., Muller-Enoch D and Blair I.A. *Oxidation of quinidine by human liver cytochrome P450.* Mol. Pharmacol. **1986**; 30: 287-295.

Gut J., Catin T., Dayer P, Kronbach T, Zanger U. *Debrisoquine/sparteine-type polymorphism of drug oxidation.* J. Biol. Chem. **1986**; 261: 11734-11743.

Guttendorf R.J., Britto M., Blouin R.A., Foster T.S., John W., Pittman K.A. and Wedlund P.J. *Rapid screening for polymorphisms in dextromethorphan and mephenytoin metabolism.* Br. J. Clin. Pharmac. **1990**; 29: 373-380.

Hammer C.G., Holmstedt B and Ryhage R. *Mass fragmentography. Identification of chlorpromazine and its metabolites in human blood by a new method.* Anal. Biochem. **1968**; 25: 532-548.

Hammer W. and Sjoqvist F. *Plasma levels of monomethylated tricyclic antidepressants during treatment with imipramine-like compounds.* Life Sci. **1967**; 6: 1895-1903.

Hegadoren K., Baker G.B. and Coutts R.T. *Interactions of iprindole with fenfluramine metabolism in rat brain and liver.* J. Psychiatr. Neurosci. **1991**; 16: 5-11.

Henthron T.K., Benitez J., Avram M.J. Matrinez C., Llerena A., Cobaleda J., Krejcie T.C. and Gibbons R.D. *Assessment of the debrisoquine and dextromethorphan phenotyping tests by gaussian mixture distributions analysis.* Clin. Pharmacol. Ther. **1989**; 45: 328-333.

Hordern A. *The antidepressant drugs.* New Eng. J. Med. **1965**; 272: 1159-1169.

Horwell D.C. *Drugs in central nervous system disorders.* In: Clinical Pharmacology. Weiner M, ed. Mercel Dekker, Inc., New York. **1985**: pp. 70-121.

Hughes H.B., Biehl J.P. and Jones A.P. *Metabolism of isoniazid in man as related to the occurence of peripheral isoniazid neuritis.* Am. Rev. Respir. Dis. **1954**; 70: 266-273.

Idle J.R. and Smith R.L. *Polymorphisms of oxidation at carbon centers of drugs and their clinical significance.* Drug Metab. Rev. **1979**; 9: 301-317.

Inaba T., Jurima M., Mahon W.A. and Kalow W. *In vitro studies of two isozymes of human liver cytochrome P450. Mephenytoin p-hydroxylase and sparteine monooxygenase.* Drug Metab. Disp. **1985**; 13: 443-448.

Islam S.I., Idle J.R.,Smith R.L. *The polymorphic 4-hydroxylation of debrisoquine in a Saudi-Arab population.* Xenobiotica. **1980**; 10: 819-825.

Jacqz E., Goto F., Nakamura K., Wilkinson G.R. and Branch R.A. *Interethnic differences in polymorphism of s-mephenytoin hydroxylation between Japanese and Caucasians.* Clin. Pharmacol. Ther. **1985**; 37: 202.

Jacqz E., Hall S.D. and Branch R.A. *Genetically determined polymorphisms in drug oxidation.* Hepatology. **1986**; 6: 1020-1032.

John Wyeth & Brother Limited, Berks (eds.). *Prondol:*A Wyeth technical booklet. **1972**:2

Kaisary A., Smith P., Jacqz E., McAllister C.B.,Wilkinson G.R., Ray W.A. and Branch R.A. *Genetic predisposition to bladder cancer: ability to hydroxylate debrisoquine and mephenytoin as risk factors.* Cancer Res. **1987**; 47: 5488-5493.

Kalow W. *Ethnic differences in drug metabolism.* Clin. Pharmacokinet. **1982**; 7:373-400.

Kalow W. *Genetics of drug transformation.* Clin. Biochem. **1986**; 6:76-82.

Klaassen C.D., Eaton D.L. and Cagen S.Z. *Hepatobiliary disposition of xenobiotics.* In: Progress in Drug

Metabolism. Vol. 6. Bridges J.W and Chasseaudd L.F. eds., John Wiley and Sons Ltd., Toronto. **1981**; pp. 1-75.

Klerman G.L. *Antidepressant drug therapy: A historical perspective.* Psychopharmacol. Bull. **1984**; 20: 211-212.

Kline B.J. and Soine W.H. *Gas chromatography: Theory, instrumentation, and pharmaceutical applications.* In: Pharmaceutical Analysis. Modern Methods. Part A. Munson J.W. ed., Marcel Dekker Inc., New York. **1984** ;pp. 1-103.

Klippert P.J.M. and Noordhoek J. *Influence of administration route and blood sampling site on the area under the curve: Assessment of gut wall, liver and lung metabolism from a physiological model.* Drug Metab. Dispos. **1983**; 11: 62-66.

Knapp D.R. Handbook of Analytical Derivatization Reactions. John Wiley & Sons, New York, **1979**.

Kobayashi S., Murray S., Waston D., Sesardic D., Davies D.S. and Boobis A.R. *The specificity of inhibition of debrisoquin 4-hydroxylase activity by quinidine and quinine in the rat is the inverse of that in man.* Biochem. Pharmacol. **1989**; 38: 2795-2799.

Koppel C., Tenczer J. and Ibe K., *Urinary metabolites of dextromethorphan in man.* Arzneim-Forsch./Drug Res. **1987**; 37: 1304-1306.

Koppel C.and Tenczer J. *Gas chromatographic-mass spectrometric study of the urinary metabolism of trimipramine.* J. Chromatogr. **1988**; 431: 197-202.

Krogh C.M.E., Gillis M.C., Bisson R and Blais D. eds. Compendium of Pharmaceuticals and Specialties (CPS). 26th edition, **1991**.

Kupfer A. and Branch R.A. *Stereoselective mephobarbital hydroxylation cosegregates with mephenytoin hydroxylation.* Clin. Pharmacol. Ther. **1985**; 38: 414-418.

Kupfer A. and Preisig R. *Pharmacogenetics of mephenytoin: A new drug hydroxylation polymorphism in man.* Eur. J. Clin. Pharmacol. **1984**. 26: 753-759.

Kupfer A., Schmid B. and Preisig R. *Dextromethorphan as a safe probe for debrisoquine hydroxylation polymorphism.* Lancet. **1984**; 2: 517-518.

Lahti R.A. and Maickel R.P. *The tricyclic antidepressants-Inhibition of norepinephrine uptake as related to potentiation of norepinephrine and clinical efficacy.* Biochem. Pharmacol. **1971**; 20: 482-486.

Lapierre Y.D. *A review of trimipramine:30 years of clinical use.* Drugs. **1989**; 38:17-24.

Li PP, Warsh JJ, Stanacev NZ *In vitro and ex vivo effects of antidepressants on rat brain membrane-bound phosphatidylinositol synthetase activity.* Neurochem Res. **1988**;13:789-795.

Linnoila M., Insel T., Kilts C., Potter W.Z. and Murphy D.L. *Plasma steady-state concentrations of hydroxylated metabolites of clomipramine.* Clin. Pharmacol. Ther. **1982**; 32: 208-211.

Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J. *Protein measurement with the Folin phenol reagent.* J. Biol. Chem. **1951**; 193: 265-275.

Lu A.H. and West S.B. *Reconstituted mammalian mixed function oxidases: requirements, specificities and other properties.* Pharmac. Ther. **1975**; A2: 337-358.

Maas J.W. *Biogenic amines and depression.* Arch. Gen. Psychiat. **1975**; 32: 1357-1361.

MacFarlene J.G., Jalali S. and Grace E.M. *Trimipramine in rheumatoid arthritis: a randomized double-blind trial in relieving pain and joint tenderness.* Curr. Med. Res. Opin. **1986**; 10:89-93.

MacKay H.P., Piskard W.R. Mitchell K.G. and Crean G.P. *A double-blind study of trimipramine in the treatment of active duodenal ulceration.* Scand. J. Gastroenterol. **1984**; 19: 190-193.

Mahgoub A., Idle J.R., Dring L.G., Lancaster R. and Smith R.L. *Polymorphic hydroxylation of debrisoquin in man.* Lancet. **1977**; 2: 584-586.

Mahgoub A., Idle J.R., Smith R.L. *A population and family study of the defective alicyclic hydroxylation of debrisoquine among Egyptians.* Xenobiotica. **1979**; 9: 51-56.

Mannering G.J. *Microsomal enzyme systems which catalyze drug metabolism.* In: Fundamentals of Drug Metabolism and Disposition. La Du B.N., Mandel H.G. and Way E.L., eds. The Willliams and Wilkins Company, Baltimore. **1971**: 206.

Martin L.E. and Reid E. *Isolation of drug metabolites.* In: Progress in Drug Metabolism. Vol. 6. Bridges J.W and Chasseaudd L.F. eds., John Wiley and Sons Ltd., Toronto. **1981**; pp. 197-248.

Mason H.S. *Oxidases.* Ann. Rev. Biochem. **1965**; 34: 595-634.

Maurer H and Pfleger K. *Screening procedure for detection of antidepressants and their metabolites in urine using a computerized gas chromatographic-Mass spectrometric technique.* J. Chromatogr. **1984**; 305: 309-323.

Maurer H. *Metabolism of trimipramine in man.* Arzneimittel Forschung:Drug Res. **1989**; 39: 101-103.

Mazel P. General *principles and procedures for drug metabolism in vitro.* In: Fundamentals of Drug Metabolism and Drug Disposition. La Du B.N., Mandel H.G. and Way E.L., eds. The Willliams and Wilkins Company, Baltimore. **1971**; pp.527-545.

McEvoy G.K., Litvak K. and Douglas P.M. eds. American Hospital Formulary Service. Drug Information. American Society of Hospital Pharmacists. **1991**

McKenna K.F., Drebit R., Hussain M.S. and Coutts R.T. *The role of cytochrome P450IID6 in the metabolism of tricyclic antidepressants.* Abstract #29e. Proc. 41st. Ann. Meet. Can. Psychiat. Assoc. **1991**; pp 26.

McTavish D. and Benfield P. Clomipramine: *An overview of its pharmacological properties and a review of its therapeutic use in obsessive compulsive disorders and panic disorders.* Drugs. **1990**, 39: 136-153.

Meyer U.A., Gut J., Kronbach T., Skoda C., Meier U.T. and Catin T. *The molecular mechanism of the common polymorphisms of drug oxidation evidence for functional changes in cytochrome P450 isozymes catalyzing bufuralol and mephenytoin oxidation.* Xenobiotica. **1986**; 16: 449-464.

Meyer U.A., Skoda R.C. and Zanger M.U. *The genetic polymorphism of debrisoquine/sparteine metabolism-molecular mechanism.* Pharmac. Ther. **1990**; 46: 297-308.

Millard B.J. *Newer developments in the mass spectrometry of drugs and metabolites.* In: Progress in Drug Metabolism. Bridges J.W and Chasseaud L.F. eds., John Wiley & Sons Ltd., New York. **1976**; pp.1-40.

Miller G.L. *Protein determination for large numbers of samples.* Anal. Chem. **1959**; 31: 964.

Miller K.W., Freeman J.J., Dingell J.V. and Sulser F. *On the mechanism of amphetamine potentiation by iprindole.* Experienta (Basel). **1970**; 26: 281-291.

Moffat A.C., Jackson J.V., Moss M.S. and Widdop B. Clark's Isolation and Identification of drugs. **1986**; pp. 1051-1052.

Moody D.E., Ruangyuttikarn W. and Law M.Y. *Quinidine inhibits in vivo metabolism of amphetamine in rats: Impact upon correlation between GC/MS and immunoassay findings in rat urine.* J. Anal. Toxicol. **1990**; 14: 311-317.

Mosnaim A.D., Callaghan O.H. and Wolf M.E. *Hydroxylation of phenylethylamine by rat liver microsomes: Inhibition studies.* Gen. Pharmacol. **1989**; 20: 463-467.

Mudler G.H., Scholtens E. and Meuer D.K.F. *Collection of metabolites in bile and urine from rat.* Methods Enzymol. **1981**; 77: 21-30.

Mueller G.C. and Miller J.A. *The metabolism of methylated aminoazo dyes. II. Oxidative demethylation by rat liver homogenates.* J. Biol. Chem. **1953**; 202: 579-587.

Mueller G.C. and Miller J.A. *The reductive cleavage of 4-dimethylaminoazobenzene by rat liver: the intracellular distribution of the enzyme system and its requirement for triphosphopyridine nucleotide.* J. Biol. Chem. **1949**; 180: 1125-1136.

Muralidharan G., Hawes E.M., Korchinski E.D. and Midha K.K. *Quinidine but not quinine inhibits the metabolism of methoxyphenamine in vivo in man.* Pharm. Res. **1989**a; 6: S-208.

Muralidharan G., Midha K.K., McKay G., Hawes E.M. and Inaba T. *Selective in vivo inhibition by quinidine of methoxyphenamine oxidation in rat models of human debrisoquine polymorphism.* Xenobiotica. **1989**b; 19: 189-197.

Murray M. *Mechanisms of the inhibition of cytochrome P450-mediated drug oxidation by therapeutic agents.* Drug Metab. Rev. **1987**; 18: 55-81.

Nagy A. and Hansen T. *The kinetics of imipramine-N-oxide in man.* Acta Pharmac. toxicol. **1978**; 42: 58-67.

Nagy A. and Johansson R. *The demethylation of imipramine and clomipramine as apparent from their plasma kinetics.* Psychopharmacol. **1977**; 54: 125-131.

Nakamura K., Goto F., Fay W.A., McAllister C.B., Jacqz E., Wilkinson G.R. and Branch R.A. *Interethnic differences in genetic polymorphism of debrisoquine and mephenytoin hydroxylation between Japanese and Caucasian populations.* Clin. Pharmacol Ther. **1985**; 38: 402-408.

Narasimhachari N., Saady J. and Friedel R.O. *Quantitative mapping of metabolites of imipramine and desipramine in plasma samples by gas chromatography-mass spectrometry.* Biol. Psychiat. **1981**; 16: 937-944.

Narasimhachari N., Saady J.J., Joseph A., Ettigi P. and Friedel R.O. *Evidence of in vivo methylation of the secondary amino antidepressant desipramine to imipramine in humans.* J. Clin. Psychopharmacol. **1982**; 2: 413-416.

Nebert D.W. and Gonzalez F.J. *P450 genes: Structures, evolution, and regulation.* Ann. Rev. Biochem. **1987**; 56: 945-993.

Nebert D.W., Nelson D.R., Adesnik M., Coon M.J., Estabrook R.W., Gonzalez F.J., Guengerich F.P., Gunsalus, I.C., Johnson E.F., Kemper B., Levin W., Phillips I.R., Sato R. and Waterman M.R. *The P450 superfamily: Updated listing of all genes and recommended nomenclature for the chromosomal loci.* DNA. **1989**; 8:1-13.

Netter K.J. *Inhibition of oxidative drug metabolism in microsomes.* Pharmacol. Ther. **1980;** 10: 515-535.

Oguri K., Yamada-Mori I. Shigezane J., Hirano T.A. and Yoshumura H. *Potentiation of physical dependance by conjugation at the 6 position of nalorphine.* Eur. J. Pharmacol. **1984**; 102: 229-235.

Okey A.B. *Enzyme induction in the cytochrome P450 system.* Pharmac. Ther. **1990**; 45: 241-298.

Omura T and Sato R. *The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification and properties.* J. Biol. Chem. **1964**. 239: 2379-2385.

Orrenius S., Ellin A., Jakobsson S.V., Thor H., Cinti D.L., Schenkman J.B. and Estabrook R.W. *The cytochrome P450 containing mono-oxygenase system of rat kidney cortex microsomes.* Drug. Metab. Disp. **1973**; 1: 350-357.

Orsulak P.J. *Therapeutic monitoring of antidepressant drugs: Guidelines updated.* Ther. Drug Monitoring. **1989**; 11: 497-507.

Otton S.V., Inaba T and Kalow W. *Competitive inhibition of sparteine oxidation in human liver by beta-adrenoceptor antagonists and other cardiovascular drugs.* Life Sci. **1984**; 34: 73-80.

Pearson J.D.M. and Sharman D.F. *The estimation of 3,4-dihydroxyphenylacetic acid, homovanillic acid and homo-isovanillic acid in nervous tissue by gas-liquid chromatography and electron capture detection.* Br. J. Pharmac. **1975**; 53: 143-148.

Perel J.M., Stiller R.L. and Pollock B.G. *Pharmacokinetic studies on oral and intravenous clomipramine.* Clin. Chem. **1986**; 32: 1059.

Populaire P., Terlain B., Pascal S., Lebreton G. and Decouvelaere B. *Absorption, excretion and biotransformation of trimipramine in dog and rabbit.* Prod. Pharm. **1970**; 25: 632-645.

Potter W.Z. *Active metabolites of tricyclic antidepressants.* In: Clinical Pharmacology in Psychiatry. Neuroleptic and Antidepressant Research. Usdin E., Dahl S.G., Gram L.F. and Lingjaerde O., eds. MacMillan, London. **1981**: 139-153.

Potter W.Z. and Calil H.M. *Metabolites of tricyclic antidepressants: biological activity and clinical implications.* In: Clinical Pharmacology in Psychiatry. Usdin E. ed, Elsevier, New York. **1981**: 311-324.

Potter W.Z. and Manji H.K. *Antidepressants, metabolites, and apparent drug resistance.* Clin. Neuropharmacol. **1990**; 13: S45-S53.

Potter W.Z., Calil H.M., Manian A.A., Zavadil A.P. and Goodwin F.K. *Hydroxylated metabolites of tricyclic antidepressants: preclinical assessment of activity.* Biol. Psychiat. **1979**; 14: 601-613.

Potter W.Z., Calil H.M., Sutfin T.A. Zavadil A.P., Jusko W.J., Rapoport J. and Goodwin F.K. *Active metabolites of imipramine and desipramine in man.* Clin. Pharmacol. Ther. **1982**; 31: 393-401.

Prien F.P. and Blaine J.D. *Workshop report-Antidepressant Drug Therapy: The role of the New Antidepressants: Summary and Conclusion.* Psychopharmacol. Bull. **1984**; 20: 295-302.

Prox A.,and Breyer-Pfaff U. *Amitriptyline metabolites in human urine. Identification of phenols, dihydrodiols, glycols and ketones.* Drug Metab. Disp. **1987**; 15: 890-896.

Randrup A. and Braestrup C. *Uptake inhibition of biogenic amines by newer antidepressant drugs: relevance to the dopamine hypothesis of depression.* Psychopharmacol. **1977**; 53: 309-314.

Reed K.L. *Gas chromatographic-mass spectrometric analysis of antidepressants, neuroleptics, and benzodiazepines.* In: Neuromethods 10: Analysis of Psychiatric Drugs. Boulton A.A, Baker G.B. and Coutts R.T. eds., Humana Press, Clifton, New Jersey. **1988**; pp. 99-154.

Reidman M. *Gas chromatographic analysis of drugs and drug metabolites in biological samples.* Xenobiotica. **1973**; 3: 411-434.

Remmer H and Merker H.J. *Drug-induced changes in the liver endoplasmic reticulum: association with drug-metabolising enzymes.* Science. **1963**; 142: 1657-1658.

Remmer H. *The role of liver in drug metabolism.* Am. J. Med. **1970**; 49: 617-629.

Reynolds J.E.F., Parfitt K., Parson A.V. and Sweetman S.C. (eds.) Martindale The Extra Pharmacopoeia. 29th edition. The Pharmaceutical Press, London. **1989**; pp. 383.

Reynolds J.E.F.(eds.) Martindale The Extra Pharmacopoeia. 28th edition. The Pharmaceutical Press, London. **1982**; pp. 133.

Richelson E. and Nelson A. *Antagonism by antidepressants of neurotransmitter receptors of normal human brain in vitro.* J. Pharmacol. Exp. Ther. **1984**; 230: 94-102.

Richelson E. *The newer antidepressants: Structures, pharmacokinetics, pharmacodynamics, and proposed* *mechanisms of action.* Psychopharmacol. Bull. **1984**; 20: 213-223.

Robbins D.K. and Wedlund P.J. *A pharmacogenetic screening experiment demonstrating principles of genetic constitution on drug metabolism.* Am. J. Pharmac. Edu. **1990**; 4: 243-249.

Robinson D.S. *Adverse reactions, toxicities and drug interactions of newer antidepressants: Anticholinergic, sedative and other side effects.* Psychopharmacol. Bull. **1984**; 20: 280-290.

Rollins D., Alvan G., Bertilsson L., Gillette J.R., Mellstrom B., Sjoqvist F. anf Traskman L. *Interindividual differences in the demethylation of amitriptyline.* Clin. Pharmac. Ther. **1980**; 28: 121-129.

Rudorfer M.V. and Potter W.Z. *Metabolism of drugs used in affective disorders.* In: Pharmacotherapy of Affective Disorders: Theory and Practice. Dewhurst W.G. and Baker G.B. eds, New York University Press. **1985**; pp. 382-448.

Rudorfer M.V. and Potter W.Z. *Pharmacokinetics of antidepressants.* In: Psychopharmacology: The Third Generation of Progress. Meltzer H.Y. ed., Raven Press, New York. **1987**; 1353-1363.

Rudorfer M.V. and Robins E. *Fatal nortriptyline overdose, plasma levels, and in vivo methylation of tricyclic antidepressants.* Am. J. Psychiatry. **1981**; 138: 982-983.

Sallee F.R. and Pollock B.G. *Clinical pharmacokinetics of imipramine and desipramine.* Clin. Pharmacokinet. **1990**; 18: 346-364.

Satterwhite J.H. and Boudinot F.D. *Effects of age and dose on the pharmacokinetics of ibuprofen in the rat.* Drug Metab. Dispos. **1991**; 19: 61-67.

Schmid B., Bircher J., Preisig R and Kupfer A. *Polymorphic dextromethorphan metabolism: Co-segregation of oxidative O-demethylation with debrisoquine hydroxylation.* Clin. Pharmacol. Ther. **1985**; 38: 618-624.

Scoggins B.A., Maguire K.P., Norman T.R. and Burrows G.D. *Measurement of tricyclic antidepressants, part I: a review of methodology.* Clin. Chem. **1980a**; 26: 5-17.

Sedlock M.L., Ravitch J. and Edwards D.J. *The effect of imipramine and iprindole on the metabolism of octopamine in the rat.* Neuropharmacol. **1985**; 24: 705-708.

Shahi G.S., Das N.P. and Moochhala S.M. *Parkinson's disease and cytochrome P450: A possible link?.* Medical Hypotheses. **1990**; 32: 277-282.

Shahidi N.T. *Acetophenetidin-induced methemoglobinemia.* Ann. N. Y. Acad. Sci. **1968**; 151: 832.

Shu Y-Z., Hubbard J.W., McKay G., Hawes E.M. and Midha K.K. *Cis-flupentixol metabolites in rat plasma and bile. The first proof of glutathione conjugation at the exocyclic double bond.* Drug Metab. Dispos. **1991**; 19: 154-162.

Sisenwine S.F., Tio C.O. and Ruelius H.W. *The disposition of [^{14}C]iprindole in man, dog, miniature swine, rhesus monkey and rat.* Xenobiotica. **1979**; 9: 237-246.

Sisenwine S.F., Tio C.O. Shrader S.R. and Ruelius H.W. *The biotransformation of protriptyline in man, pig and dog.* J. Pharmacol. Exp. Therap. **1970**; 175: 51-59.

Sjoqvist F., Berglund F., Borga O, Hammer W. and Anderson S. *The pH dependent excretion of monomethylated tricyclic antidepressants in dog and man.* Clin. Pharmacol. Ther. **1969**; 10: 826-833.

Spina E., Steiner E., Dumont E. and Dahlqvist R. *Inhibition of desipramine 2-hydroxylation by quinidine and quinine in rapid and slow debrisoquine hydroxylators.* Psychopharmacol. Ser. **1989**; 7: 201-201.

Srinska V. Ohman J and Wellstead C. *Gas chromatography-mass spectrometry of maprotiline in serum.* Clin. Chem. **1984**; 30: 1276-1277.

Steiner E., Dumont E., Spina E. and Dahlqvist R. *Inhibition of desipramine 2-hydroxylation by quinidine and quinine.* Clin. Pharmacol. Ther. **1988**; 43: 577-581.

Steranka L.R. *Long-term decreases in striatal dopamine, 3,4-dihydroxyphenylacetic acid and homovanillic acid after a single injection of amphetamine in iprindole-treated rats: time course and time-dependent interactions with amfonelic acid.* Brain Res. **1982**; 234: 123-126.

Stinson S.C. *Psychoactive Drugs.* Chemical and Engineering News. **1990**; October 15 issue: 33-68.

Suckow R.F. and Cooper T.B. *Determination of trimipramine and metabolites in plasma by liquid chromatography with electrochemical detection.* J. Pharm. Sci. **1984**; 73: 1745-1748.

Sutfin T.A., DeVane C.L. and Jusko W.J. *The analysis and disposition of imipramine and its active metabolites in man.* Psychopharmacol. **1984**; 82 :310-317.

Testa B and Jenner P. *Novel drug metabolites produced by functionalization reactions: Chemistry and toxicity.* Drug Metab. Rev. **1978**; 7: 325-369.

Testa B. and Jenner P. Drug Metabolism: Chemical and Biochemical Aspects. Vol.4. Marcel Dekker, New York. **1976**.

Testa B. and Jenner P. *Inhibitors of cytochrome P450s and their mechanism of action.* Drug Metab. Rev. **1981**; 12: 1-117.

Vandel B., Sandoz M., Vandel S., Allers G. and Volmat R. *Biotransformation of amitriptyline in depressive patients: urinary excretion of seven metabolites.* Eur. J. Clin. Pharmacol. **1982**; 22: 239-245.

Vandenheuvel W.J.A. and Zacchei A.G. *Gas-liquid chromatography in drug analysis.* Advances in Chromatogr. **1976**; 14: 199-263.

Vanio H. and Hietanen E. In: Aspects of Drug Metabolism. Testa B and Jenner P eds., Marcel Dekker, New York. **1980**. Vol.4.

Vessel E.S. *Genetic and environmental factors affecting drug disposition in man.* Clin. Pharm. Ther. **1973**; 22: 659-679.

Vetticaden S.J., Cabana B.E., Prasad V.K., Purich E.D., Jonkman J.H.J., de Zeeuw R., Ball L., Leeson L.J. and Braun R.L. *Phenotyping differences in dextromethorphan metabolism.* Pharm. Res. **1989**; 6: 13-19.

Vinks A., Inaba T., Otton S.V. and Kalow W. *Sparteine metabolism in Canadian Caucasians.* Clin. Pharmacol. Ther. **1982**; 31: 23-29.

von Bahr C., Groth C., Jansson H., Lundgren G., Glaumann H., Lind M. and Glaumann H. *Drug metabolism in human liver in vitro: Establishment of a human liver bank.* Clin. Pharmacol. Ther. **1980**; 27: 711-725.

von Bahr C., Spina E., Birgerson C., Ericsson O, Goransson M., Henthron T and Sjoqvist F. *Inhibition of desmethylimipramine 2-hydroxylation by drugs in human liver microsomes.* Biochem. Pharmacol. **1985**; 34: 2501-2505.

Wagner J., Northam J.F., Alway C.D. and Carpenter O.S. *Blood levels of drug at the equilibrium state after multiple dosing.* Nature. **1965**; 207: 1301-1302.

Weder H.J. and Bickel M.H. *Separation and determination of imipramine and its metabolites from biological samples by gas-liquid chromatography.* J. Chromatog. **1968**; 37: 181-189.

Williams R.T. Detoxication Mechanisms. Wiley, New York.**1959**;2

Williams R.T., Millburn P. and Smith R.L. *The influence of enterohepatic circulation on toxicity of drugs.* Ann. NY Acad. Sci. **1965**; 123: 110-124.

Woodworth J.R. Dennis S.R.K., Moore L. and Rotenberg K.S. *The polymorphic metabolism of dextromethorphan.* J Clin. Pharmacol. **1987**; 27: 139-143.

Woolhouse N.M., Andoh B., Mahgoub A., Sloan T.P., Idle J.R. and Smith R.L. *Debrisoquine hydroxylation polymorphism* *among Ghanians and Caucasians.* Clin. Pharmacol. Ther. **1979**; 26: 584-591.

Yoshimura H., Ida S., Oguri K. and Tsukamoto H. *Biochemical basis for the analgesic activity of morphine-6-glucuronide in the brain of rats.* Biochem. Pharmacol. **1973**; 22:1423-1430.

Zanger U.M., Vilbois F., Hardwick J. and Meyer U.A. *Absence of hepatic cytochrome P450buf I causes genetically deficient debrisoquine oxidation in man.* Biochemistry. **1988**; 27: 5447-5454.

Zeigler V.E., Biggs J.T., Wylie L.T., Coryell W.H., Hanifl K.M., Hawf D.J. and Rosen S.H. *Protriptyline kinetics.* Clin. Pharmacol. Ther. **1978**; 23: 580-584.

Zhou H.H., Anthony L.B., Roden D.M. and Wood A.J. *Quinidine reduces clearance of (+)-propranolol more than (-)-propranolol through marked reduction in 4-hydroxylation.* Clin. Pharmacol. Ther. **1990**; 47: 686-693.

Zis A. and Goodwin F. *Novel antidepressants and the biogenic amine hypothesis of depression.* Arch. Gen. Psychiat. **1979**; 36: 1097-1107.

APPENDIX

CURRICULUM VITAE

MOHAMMED SALEH HUSSAIN
437 Michener Park
Edmonton, Alberta
Canada T6H 4M5

PERMANENT ADDRESS	Hussain Villa, Rayhussain, Amberkhana, Sylhet, Bangladesh
EDUCATION	
Ph.D.	Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada.
Area of research	**Analysis & Drug metabolism**
M.Sc	University of Dhaka, Dhaka, Bangladesh
B.Sc.(Honors)	University of Chittagong, Chittagong, Bangladesh

COURSES TAKEN AT THE UNIVERSITY OF ALBERTA

Course #	***Course Name***	***Grade***
Pharm 630	Metabolism & Excretion of Drugs	9/9
Pharm 626	Advanced Mass Spectrometry	9/9
Pharm 624	Advanced N.M.R. Spectroscopy	AU
Pharm 574	Advanced Forensic Analysis	9/9
Pharm 573	Advanced Pharmaceutical Analysis	9/9
Pharm 570	Advanced Spectroscopy	8/9
Pharm 421	Biopharmaceutical Chemistry	AU
Pharm 420	Medicinal Chemistry	AU
Pharm 415	Biopharmaceutics and Pharmacokinetics	9/9

AWARDS AND HONORS

- ***Alberta Heritage Foundation for Medical Research full time Studentship Award (1991)***
- ***FGSR Research Travel Award (1991)***
- ***University of Alberta Bursary Award (1991)***
- ***Myer Horowitz Graduate Scholarship Award (1990) [only one awarded in the faculty]***
- ***SynPhar Graduate Scholarship (1988)***
- ***Merit Scholarship (undergraduate studies)***

ASSOCIATIONS AND COMMITTEES

- ***President,*** Pharmacy Graduate Students Association, University of Alberta (1990/91 session)
  Duties:
  - Represent pharmacy graduate students in council meetings and in faculty graduate studies committee meetings
  - Chair pharmacy graduate student's meetings
  - Organize seminars and workshops

INSTRUMENTATION

Experienced in the operation and know the techniques of

Chromatography:	Gas Chromatography (Hewlett Packard & Varian Vista Series) equipped with FID, NP and ECD detectors High Performance Liquid Chromatography (Varian)
Mass Spectrometry:	Quadropole Type:Hewlett Packard model:5980 A attached to a 5948A data system and a Tektronix monitor. Magnetic Deflection Type : Varian Vista 7070E, Analytical Organic Mass Spectrometer

equipped with computerized work station

Combined GC/MS: Hewlett Packard and Varian Vista series

NMR Spectrometry: 90 MHz (Varian EM 390) and 60 MHz (Varian EM 360A)

IR Spectrophotometry: FT-IR Nicolet DX system and Perkin Elmer 267

UV-Visible Spectrophotometry: Philips PU 8700 Series and LKB Biochrom Ultrospec II

Computer Systems : Macintosh, IBM PC and compatibles

RESEARCH EXPERIENCE

- Know the techniques of extracting, purifying, analyzing drugs and their metabolites in biological fluids and tissues
- Have expertise in the interpretation of mass spectra of drugs and other xenobiotics and their metabolites
- Know the techniques of both in vitro and in vivo drug metabolism studies
- Have experience in cannulation of jugular vein and bile duct in rat and in the preparation of liver (human, rat, guinea pig etc) microsomes
- Attended courses in handling and care of experimental laboratory animals (rat, mouse, guinea pig and dog) organized by the Health Sciences Laboratory animal services, University of Alberta, Edmonton, Canada and obtained certificates

- Performed quantitative analysis of specimens and parenteral solutions used in clinical studies at the University of Alberta Hospitals
- Developed a sensitive and rapid method of screening individuals for polymorphism in dextromethorphan metabolism. This phenotyping information is routinely used by Dr. K. McKenna et. al, department of Psychiatry, University of Alberta Hospitals, for rational antidepressant drug therapy

INDUSTRIAL EXPERIENCE

- Worked as a senior Quality Control Chemist in a Pharmaceutical Industry in Bangladesh
  Duties:
  - managed and supervised laboratory
  - in charge of quality control of raw materials and finished products
  - assisted sales representatives in promoting sales
- Attended Quality Control Courses organized by the Drugs Administration, Bangladesh in collaboration with Glaxo (Bangladesh) Ltd, Pfizer (Bangladesh) Ltd, and Hoechst (Bangladesh) Ltd. Obtained certificate
- Currently working as a post doctoral scientist in the Drug Metabolism Division of SynPhar Laboratory Inc., Edmonton,Alberta

TEACHING EXPERIENCE

- Taught Pharm 630 (Drug Metabolism) course (2nd term, 1991/1992 session) to graduate students of the faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta
  Topics taught:
  1. Enzyme activation and inhibition: Environmental factors in drug metabolism
  2. Drug-drug interactions: influence on drug metabolism

- Graduate Teaching Assistant, Faculty of Pharmacy & Pharmaceutical Sciences, University of Alberta:
    - Lab. instructor : Pharm 570 (Advanced Pharmaceutical Analysis- graduate course)
      Duties: Preparation and demonstration of lab experiments; supervision and evaluation of laboratory assignments.
    - Teaching Assistant: Pharm 320 (Medicinal Chemistry)
      Duties: Evaluate assignments
    - Lab Instructor: Pharm 325 (Pharmaceutical Analysis)
      Duties: Preparation and supervision of laboratory work
- Attended seminars in improving teaching skills, organized by CITL, University of Alberta, Edmonton, Canada.
- Wrote grant applications to AHFMR and MRC for summer studentships
- Supervised summer students in their research work in the University of Alberta
- Worked as a lecturer in Chemistry in two educational institutions in the Cross River State of Nigeria, West Africa
  Duties: Teach 'O' and 'A' level chemistry. Organised both internal and external examinations and acted as the head of the chemistry department for sometime
- Supervisor and examiner of the West African Examinations Council, Lagos, Nigeria, West Africa
  Duties: Evaluate and supervise both 'O' and 'A' level examinations.
- Worked as a lecturer in chemistry in a college in Bangladesh
  Duties: Teach undergraduate chemistry students

PROFESSIONAL QUALIFICATION

Registered Pharmacist with the Pharmacy Council of Bangladesh

PROFESSIONAL AFFILIATIONS

- ***American Association of Pharmaceutical Scientists : Member***
- ***American Society for Mass Spectrometry : Member***
- ***Pharmacy Council of Bangladesh : Member***
- ***The Chemical Institute of Canada: Member***
- ***Canadian Society for Chemistry: Member***

HOBBIES

- Travelling : Travelled a few countries in Africa, Asia, Europe and North America
- Photography
- Playing Tennis, Chess and Contract Bridge

PUBLICATIONS

PAPERS

1. RT Coutts, **MS Hussain**, RG Micetich, GB Baker & M Daneshtalab.(**1990**). The metabolism of trimipramine in the rat, *Biomed. & Environ. Mass Spectrometry,* 19, 793-806.

2. **MS Hussain**, RT Coutts, GB Baker, RG Micetich & M Daneshtalab. (**1991**). The metabolism of antidepressants: Urinary metabolites of trimipramine in the rat, *Prog.Neuropsycho-pharmacol & Biol. Psychiat.*, 15, 285-289.

3. RT Coutts, **MS Hussain**, RG Micetich, GB Baker & M Daneshtalab. (**1991**). Structural characterization of the urinary metabolites of Iprindole in rat, *Xenobiotica* , 21,1393-1405.

4. RT Coutts, **MS Hussain** and GB Baker. (**1991**). Effect of iprindole on the metabolism of trimipramine in the rat, *J. Psychiatr. Neurosci.*, 16, 272-275.

5. RT Coutts, GB Baker, F. Malek and **MS Hussain**. (**1991**). Aromatic oxidation of N-*n*-butylamphetamine is enhanced in the rat by prior administration of quinidine. *Res. Commun. Chem. Pathol. Pharmacol.*, 74, 15-24.

MANUSCRIPT UNDER PREPARATION:

6. **MS Hussain,** RT Coutts et. al. Metabolism of trimipramine in man : evidence of alicyclic hydroxylation.
7. **MS Hussain**, RT Coutts et. al. Structural characterization of the biliary metabolites of trimipramine in the rat : evidence of glucuronidation and sulfate formation.

8. **MS Hussain**, RT Coutts et. al. Structural characterization of the biliary metabolites of iprindole and imipramine in the rat.
9. **MS Hussain**,RT Coutts et, al. Quantitation of the major metabolites of imipramine in man.
10. **MS Hussain**, RT Coutts et. al. Effects of quinidine and quinine on the metabolism of trimipramine in the rat.
11. **MS Hussain**, RT Coutts et. al. Utility of acetylation in the identification of drug metabolites.

ABSTRACTS

1. **MS Hussain**. (**1990**). Metabolic oxidation of first generation tricyclic antidepressant, abstract #5, ***Pharm 699 graduate seminar***, Faculty of pharmacy & Pharmaceutical Sciences, University of Alberta.

2. RT Coutts, **MS Hussain**, RG Micetich, GB Baker & M Daneshtalab. (**1990**), abstract #39, The utility of acetylation in the identification of drug metabolites, ***Proc. Research symposium & 37th Can. Conf. Pharm. Res. , Assoc. Fac. Pharm. Can. ,*** [Regina, Saskatchewan]; published in ***Clinical & Investigative Medicine***.

3. **MS Hussain**, RT Coutts, GB Baker, RG Micetich & M Daneshtalab. (**1990**), abstract #60, The metabolism of trimipramine in the rat, ***Proc. 13th Ann. Meet. Can. Coll. Neuropsychopharmacol.***, [Banff, Alberta].

4. RT Coutts, **MS Hussain**, RG Micetich, GB Baker & M Daneshtalab. (**1990**), abstract # 68, Metabolism of trimipramine and iprindole in the rat, ***Proc. 3rd North American Meet. International Soc. Study of Xenobiotics,*** [San Diego, USA].

5. **MS Hussain**, RT Coutts, RG Micetich, GB Baker & M Daneshtalab. (**1990**), abstract #PPDM 8062, Effects of

quinidine and quinine on the metabolism of trimipramine in the rat, ***Pharmaceutical Research***, 7(9), S-221, [Las Vegas, USA].

6. **MS Hussain**, RT Coutts, RG Micetich, GB Baker & M Daneshtalab. (**1990**), abstract#PPDM 8063, In vivo metabolism of iprindole in the rat, **Pharmaceutical Research**, 7(9), S-221, [Las Vegas, USA].

7. **MS Hussain**, RT Coutts, KF McKenna, M Daneshtalab & RG Micetich. (**1991**), abstract #W-1, Metabolism of imipramine in man, ***Proc. 14th Ann. Meet. Can. Coll. Neuropsychopharmacol.***, [Hamilton, Ontario].

8. RT Coutts, **MS Hussain** GB Baker, M Daneshtalab & RG Micetich. (**1991**), abstract # 25, Metabolic studies on iprindole, ***Proc. Res. Symp. & 38th Can. Conf. Pharm. Res. , Assoc. Fac. Pharm. Can.***, [St. John's, Newfoundland, Canada].

9. RT Coutts, **MS Hussain** and GB Baker. (**1991**), abstract #W-14, Effect of iprindole on the metabolism of trimipramine in the rat, ***Proc. 14th Ann. Meet. Can. Coll.Neuro- psychopharmacol.***, [Hamilton, Ontario].

10. KF Mckenna, R. Debrit, **MS Hussain** and RT Coutts. (**1991**), abstract #29e, The role of cytochrome P450IID6 isozyme in the metabolism of tricyclic antidepressants, ***Proc. 41st. Ann. Meet. Can. Psychiat. Assoc.***, [Saskatoon, Saskatchewan, Canada].

11. **MS Hussain**, RT Coutts KF McKenna, RG Micetich and M Daneshtalab (**1991**), Metabolism of trimipramine in man : evidence of alicyclic hydroxylation, abstract #PPDM 8052, ***Proc. 6th Annual meeting & exposition of the American Association of Pharmaceutical Scientists***,

Pharmaceutical Research, 8(10), S-238 [Washington DC, USA].

12. **MS Hussain**, RT Coutts GB Baker, M Daneshtalab and RG Micetich (**1991**), abstract #PPDM 8009, Biliary metabolites of trimipramine in the rat , **Proc. *6th Annual meeting & exposition of the American Association of Pharmaceutical Scientists*, *Pharmaceutical Research***, 8(10), S-228 [Washington DC, USA].

13. RT Coutts, **MS Hussain** and GB Baker (**1991**), Structural characterization of the biliary metabolites of iprindole in the rat, abstract #8013, **Proc. *6th Annual meeting & exposition of the American Association of Pharmaceutical Scientists*, *Pharmaceutical Research***, 8(10), S-229 [Washington DC, USA].

14. **MS Hussain** and RT Coutts (**1991**), In vivo metabolic studies on trimipramine, abstract #8019, **Proc. *6th Annual meeting & exposition of the American Association of Pharmaceutical Scientists*, *Pharmaceutical Research***, 8(10), S-230 [Washington DC, USA].

15. Z.A. Samnani, **M.S. Hussain** and R.T. Coutts (**1991**). Effects of quinidine on the metabolism of N-*n*-butylamphetamine in the rat, abstract #16, ***Research Day,*** Faculty of Pharmacy & Pharmaceutical Sciences, Bernard Snell Hall, Walter C. McKenzie Center, University of Alberta Hospitals [Edmonton, Alberta].

PODIUM (PLATFORM) PRESENTATIONS AT MAJOR MEETINGS

1. ***American Association of Pharmaceutical Scientists***, 5th annual meeting & exposition, Las Vegas, NV, USA, November 1990, "Effects of quinidine and quinine on the metabolism of trimipramine in the rat."

2. **American Association of Pharmaceutical Scientists**, 5th annual meeting & exposition, Las Vegas, NV, USA, November 1990, "In vivo metabolism of iprindole in the rat."

3. **Research Day,** Faculty of Pharmacy & Pharmaceutical Sciences, Bernard Snell Hall, Walter C. McKenzie Center, University of Alberta Hospitals, October 1990, "Metabolism of Iprindole and trimipramine in the rat."

4. **Association of the Faculty of Pharmacy of Canada & Canadian Pharmaceutical Association**, 37th Canadian conference on pharmaceutical research, Regina, Saskatchewan, May 1990, "The utility of acetylation in the identification of drug metabolites. "

5. **Pharm 699 graduate seminar,** Faculty of Pharmacy & Pharmaceutical Sciences, University of Alberta, Edmonton, January 1990, "The metabolic oxidation of first generation tricyclic antidepressant"- was the **Master of Ceremonies** of the occasion.

6. **Master of Ceremonies : Pharm 699 graduate seminar,** Faculty of Pharmacy & Pharmaceutical Sciences, University of Alberta, Edmonton, March, 1991.

RESEARCH INTERESTS

My major research interests include:

- Isolation, purification and structural characterization of xenobiotics (drugs, environmental pollutants etc.) and their metabolites in biological and environmental samples.

- *In vitro* and *in vivo* metabolism studies of xenobiotics.

- Stereospecific metabolism and pharmacokinetic studies of drug substrates.

- Structure and function of cytochrome P450 isozymes. These isozymes play a vital role in metabolism and thereby in the excretion of endogenous and exogenous substrates (drugs, toxins and other xenobiotics). A very important question in cytochrome P450 research is the structural basis for the particular substrate. specificity of individual cytochrome P450 isozymes.

- Purification and characterization of human cytochrome P450 isozymes.

- Identifying new substrates of clinical importance for P450IID6, P450IIC9 and P450IIIA4 isozymes.

- Polymorphism in drug metabolism and phenotyping studies.

- Metabolic induction and inhibition studies.